ATTRACTANTS AND BODY HYDROCARBON CONSTITUENTS OF THE HORN FLY, *HAEMATOBIA IRRITANS* (L.) (DIPTERA:MUSCIDAE)

By

JAMES WELDON MACKLEY

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Horn fly lipids were extracted, purified and examined for biological activity related to attraction and contact stimulation. An olfactometer was developed to assay attractancy and a modification of the "pseudofly" technique was employed to measure contact stimulation of crude and fractionated lipids. Active hydrocarbon fractions of the natural lipid were identified and the corresponding synthetic compounds were tested. Hydrocarbon components of laboratory and wild strain flies were compared.

Olfactometer assays demonstrated significant attraction ($P=.1$) of sexually mature female flies to the total monoolefins. Other lipid fractions showed no activity. Male olfactory response was irregular and produced inconclusive results.

Four major mono-olefin compounds were identified from sexually mature laboratory and wild strain flies: Z-9-tricosene, Z-5-tricosene, Z-9-pentacosene, and Z-9-heptacosene. These were tested as synthesized compounds which were identified as identical to extracted compounds. These four compounds made up 94% of the total mono-olefins in the laboratory strain flies and wild strain males. In wild strain females these compounds made up 80% of the total mono-olefins. Z-9-tricosene and Z-9-pentacosene have been identified as sex pheromones in two other muscoid species.

Paraffins were not found to be active in olfactometer assays but were chemically analyzed. The bulk of components were straight-chain molecules C_{21} to C_{29}; minor components consisted of mono- and di-methyl branched molecules and straight-chain molecules outside the C_{21} to C_{29} range. Active paraffins present in the house fly, stable fly, and tsetse fly occurred in minute quantities in the horn fly or were altogether absent.

Wild strain horn flies showed GC profiles quite different from laboratory strain flies, the most striking difference being the almost complete lack of tricosene in wild strain females. These chemical data suggest that there may be behavior differences between laboratory and wild strains.

Contact stimulation assays showed that horn fly mating activity is in part chemically mediated. Lack of response by males towards variously treated fly models when compared to response to adult females indicated that vision also plays a role in mating behavior.

INTRODUCTION

Chemical control of pest insects invariably leads to some degree of insecticide resistance and environmental pollution. The high biotic potential of most insects enables them to develop resistance to chemical poisons in a relatively short period of time. In fact, it is universally accepted in the pesticide industry that any given insecticide will not be permanently effective because insecticide resistance eventually reduces its effectiveness. For this reason, insecticide development is necessarily a continuous process, but the high cost for research, certification, and labeling of insecticides is forcing a growing number of chemical companies to abandon or reduce development in this market. Insecticides indiscriminantly used in the environment can disable or kill non-target life forms and thereby disrupt any number of the natural processes which make up our ecosystems. As contaminants in our food supply, insecticides have recently come under heavy attack because of their alleged cancer-inducing properties. FIFERA as amended is the authority by which the EPA forbids the use of carcinogens which are in any way involved in human exposure. Consequently, the production of environmently safe insecticides has placed added demands on the pesticide industry.

The horn fly is a major pest of beef and dairy cattle. As such, these latest trends in the pesticide industry and the spectre of environmental pollution are having a growing adverse impact on the prospects for its control. By law, the reserve of insecticides for use on cattle is rapidly being depleted, and industry is increasingly reluctant to provide replacements. The possibility of insecticide residues on animals restricts the available candidate compounds for beef and dairy commodities.

In an effort to reduce our reliance on insecticides, much emphasis has recently been placed on the research and implementation of "natural" forms of insect control. Some of these new approaches involve exploiting well-known behavior patterns by manipulating them to the detriment of the insect pest. One such triggered behavior pattern employed by insects is the use of chemical signals for transmitting information between individuals. The term "pheromone" has been applied to these types of chemicals and is now widely defined to include those substances secreted by an animal to influence the behavior of other animals of the same species (Wilson 1963).

Pheromones have a number of practical applications. They have been employed to increase the attractancy of insecticide and chemosterilant baits. They provide a method of estimating populations amd make possible early detection of insect infestations (Beroza and Jacobson 1963). Broadcasting a pheromone in an area of insect infestation can

disrupt the normal pattern of chemical signals, thus insects are prevented from orienting towards and locating a potential mate (Butler 1967; Gaston et al. 1967). The effects of mating disruption on a pest population are obvious. The overall result, therefore, of correct implementation of pheromones into insect control programs is a decreased dependence on insecticide use.

Since the first identification of a pheromone by Butenandt et al. (1959), a virtual flood of pheromone discoveries has followed embracing a wide variety of insect species. The recent literature is abundant, but it has been excellently reviewed and summarized by Jacobson (1972) and Mayer and McLaughlin (1975). In the family Muscidae, the first identification of a pheromone was made by Carlson et al. (1971) with the house fly, Musca domestica L. These investigators isolated a cuticular hydrocarbon, Z-9-tricosene, and demonstrated male attraction to it in laboratory olfactometers. Subsequently, Mayer et al. (1972) found a similar degree of attraction when male house flies were tested against horn fly cuticular hydrocarbons. These studies therefore prompted me to explore the possibility of chemical communication in horn flies.

The purpose of this investigation was twofold: (1) to determine the nature of pheromonal communication in horn flies and the extent to which they rely on it, and (2) to ascertain the identity of any attractants and/or sexually-based contact stimulants. To accomplish these objectives, bioassays were devised to test the activity (attractancy

and/or contact stimulation) of fractionated adult fly lipid extracts; the major components of active fractions were identified and the corresponding synthetic compounds were assayed individually or in various combinations for activity.

LITERATURE REVIEW

The Horn Fly

Distribution

The horn fly, Haematobia irritans (L.), is a native Old World species occurring from North Africa to Lapland and was first described by Linnaeus in 1758. Between 1884 and 1886, it was introduced to the United States on cattle imported from Europe. The first recorded observation in the United States was made by Riley (1889) in 1887. In little more than a decade, it had spread throughout the U.S. and much of Canada (McClintock and Depner 1954; Bruce 1964). Today, the horn fly is well established from Venezuela to Canada and in the Hawiian Islands (McClintock and Depner 1954). The characteristic heartiness and aggressiveness of the horn fly is no doubt responsible for its rapid spread and for its ability to survive extremely diverse climatic conditions.

Bionomics

Host preferences

Cattle seem to be the horn fly's principal host. However it has been known to occasionally attack sheep, goats, horses, mules, dogs, and more rarely humans (Bruce 1942,

1964). Both sexes suck blood (McClintock and Depner 1954). The adults generally congregate on the shoulders and sides of the animal, and always rest with their heads pointing downward (Bruce 1964). They are usually more numerous on black or dark-colored animals than light animals, and tend to occupy the dark areas on bi-colored animals (Burns et al. 1962; Franks et al. 1964; Morgan 1964). Bulls are preferred to cows or calves (McClintock and Depner 1954), and testosterone-treatment of steers has been shown to increase attractiveness (Dobson et al. 1970). Shorter-haired breeds and individuals are also preferred (McClintock and Depner 1954; Bruce 1964). Brahman cattle have been shown to be less attractive than the European breeds (Tugwell et al. 1969).

Life history

The adult female horn fly leaves its position on the host to oviposit in freshly passed dung (Bruce 1942, 1964; McClintock and Depner 1954; Harris 1962); larvae are not known to develop in any other natural medium (Depner 1961; Bruce 1964). The specific stimuli which induce egg laying are not known (McClintock and Depner 1954). The site of oviposition is on the underside or on the grass or soil beneath the dropping (McClintock and Depner 1954; Bruce 1964; Sanders and Dobson 1969). Bruce (1964) reported that the female fly spends up to 10 minutes to deposit 1 or more eggs, the largest number at any one time being 14. Once egg-laying is completed, the fly immediately returns to the

host. Approximately 360 eggs are produced by a female during her lifetime. Sanders and Dobson (1969) and Kunz et al. (1970) reported that females are equally active day and night in egg-laying.

Eggs hatch within 24 hours. The larvae feed and grow through 3 instars in the dropping, becoming fully developed in about 4 days (Bruce 1942, 1964; McClintock and Depner 1954). The full grown 3rd instar larvae then migrate to the underside of the dropping or into the soil and pupate; several days later the adults emerge. About one hour after eclosion, the adult attempts to fly and seek a host. Developmental lifetime is greatly influenced by temperature and moisture, thus the egg to egg life cycle can range from 9-12 days in the field (Melvin and Beck 1931; Bruce 1942, 1964; Hargett and Goulding 1962) and up to 32 days in the laboratory (Depner 1962).

Harris et al. (1971) found that eclosion of horn flies, whether reared outside or inside, followed a circadian rhythm and that variations in temperature influenced the rhythm; Hoelscher and Combs (1971b) reported a similar influence on eclosion of temperature-circadian rhythm interactions. Females emerge before males, sometimes as much as 24 hours earlier (McClintock and Depner 1954; Harris et al. 1971; Hoelscher and Combs 1971b). Horn fly sex ratios range from 1:1 to 1:1.35 (Glaser 1924; Mohr 1943).

Mating occurs on the host within 3 days of eclosion, and fertile eggs are deposited one day later (Bruce 1942,

1964; Harris et al. 1968; Schmidt 1972). Laboratory females tend to mate one day later than females held on the natural host (Harris et al. 1968). Females mate only once (Harris et al. 1968; Depner 1961); Harris et al. (1968) found that one male could inseminate 1-8 females.

Adult longevity is 6-8 weeks. Adults are found continuously on cattle from spring until late fall (Wright 1970), although populations may vary during this period. Bruce (1942, 1964) reported that warm, moist weather is most favorable to the horn fly while hot, dry weather and periods of low temperature are unfavorable. He concluded that temperature determines presence or absence and moisture determines abundance. Morgan (1964) agreed, but added that the horn fly is influenced not only by the macro-climate but the micro-climate of the hair coat mantle as well.

The adults of both sexes are obligate blood feeders. Early workers (Bruce 1942, 1964) reported that feedings occur 2 or more times a day, usually at sunrise and sunset; however flies may feed intermittently the entire day if continually interrupted. It was found that 30 minutes were required for the flies to feed to repletion. Fully fed flies increased their weights 50% over the unfed condition. Harris and Miller (1969), employing an electronic recording device in the laboratory, found female flies to feed an average of 12 times/day. The time of feeding was distributed evenly throughout the day. Feeding lasted an average of 1.2 minutes, and cumulative time spent feeding equalled 14.3 min/day.

Horn fly overwintering habits vary with geographic region. In the tropics and subtropics, breeding is known to occur throughout the winter months (Hoelscher et al. 1967). In the temperate and colder regions, the winter is spent in diapause as either a 3rd instar larva or a pupa (Depner 1962; Hoelscher et al. 1967; Kunz et al. 1972; Hoelscher and Combs 1971a). Winter diapause results from an interaction between the photoperiod to which the adults are exposed and the temperature to which the immature stages are exposed (Depner 1961, 1962; Wright 1970). Depner (1962) submitted yet another factor contributing to diapause: UV radiation received by a fly's host predisposes the pupal progeny to diapause; the host transmits a "UV factor" to adult flies which in turn transmit it to their progeny.

Flight behavior

Dispersal. Many of the earlier workers (Bruce 1938; 1942, 1964; McClintock and Depner 1954) contended that horn flies remain on their hosts continuously, the females leaving only briefly to oviposit. Eddy et al. (1962) and Hargett and Goulding (1962) reported cases where horn flies leave their hosts at night. They reasoned that the flies simply got dislodged and were unable to relocate the host in the absence of light.

Later investigators, however, disputed these points, concluding that horn flies had the ability to travel considerable distances in short periods of time. Eddy et al.

(1962) recovered marked, released horn flies up to 5 miles from release points. Tugwell et al. (1966) recovered released flies up to 1500 yds from release points within 4 hours, indicating a rather rapid host seeking capacity. Hoelscher et al. (1968) reported movement of flies between pens of animals separated by 100 yds. Kinzer and Reeves (1974) found that (1) horn flies were able to fly 7.3 miles in 10 hours to locate a host, and (2) 68-77% of the flies placed on a centrally located animal left the animal within 11 hours. Hoelscher et al. (1968) and Tugwell et al. (1966) found in their tests that greater numbers of females moved than males; Kinzer and Reeves (1974), however, found that males and females dispersed in equal numbers.

In the absence of a host, the necessity for several blood meals a day by the horn fly (McClintock and Depner 1954; Harris and Miller 1969) may demand an obligatory drive to locate a host. However, this does not explain the strong tendency for shortrange transfer between hosts (Kinzer and Reeves 1974; Hoelscher et al. 1968). The horn fly obviously possesses a very definite dispersal drive, but what triggers this drive is uncertain.

Host orientation. Through a series of carefully controlled experiments Hargett and Goulding (1962) offered as follows the probable sequence of events which orient the horn fly with respect to its host. At eclosion, light stimuli and a negative geotaxis direct the fly into the air. The fly will continue towards the light until areas of con-

trast in its visual field begin to override the light stimulus. When the fly nears areas of contrast, the specific host comes into view and a strong motor response is generated towards it. Contact with the host then results. When the horn fly leaves its host, vision must be employed to relocate it. Orientation, therefore, is greater to visual than to olfactory and heat stimulations.

The above, of course, explains only shortrange orientation. The mechanisms directing long range flights are unknown. Kinzer and Reeves (1974) reported that horn fly dispersal and host location over long range appear to be random with respect to wind direction, but directional movements were identified by temperature, wind velocity, and humidity. Time of day of release also influenced success of host location.

Damage

Direct damage

The most significant damage produced on cattle by horn flies is reduction in normal weight gain and milk production. These losses result from irritation, worry, blood loss, digestive disorders, and the general lessening of animal thrift and vigor brought on by horn fly feeding (Bruce 1942, 1964; McClintock and Depner 1954; Hoelscher and Combs 1971a). Feeding can also produce sores which may become sites of screwworm infestation (Bruce 1942, 1964). Weight losses in untreated cattle can run as high as 0.5 to 0.66 pound/animal/

day (Laake 1946; Cheng 1958; Cutkomp and Harvey 1958). Reductions in the number of calves produced has also been reported (McClintock and Depner 1954).

Numbers of flies per animal vary with respect to age, sex, and breed of the animal and with respect to locality and time of year. Generally when fly numbers exceed 400 per animal losses in some form will occur (Bruce 1964; Butler 1971). Numbers as low as 50 flies/side or 5 flies/face have been reported as economically damaging (Lofgren 1970). In Texas and Oklahoma 4,000 flies per head are not uncommon, and as many as 10,000 per head have been recorded (Bruce 1942, 1964); in Florida, numbers almost always exceed economic levels during the summer months (Butler 1970, 1971, 1972, 1973).

Disease transmission

As with any other intermittent bloodfeeding insect, the potential exists for the horn fly to vector disease. This potential was once regarded to be quite low since early workers maintained that the fly remained with its host continuously. But later studies (see section "Flight Behavior") demonstrated that the horn fly will seek out many hosts locally and is capable of extensive questing flights; thus, coupled with its bloodsucking nature, these habits create a very high potential for disease transmission (Butler et al. 1977).

Despite this potential, experimental evidence of disease transmission is lacking (McClintock and Depner 1954; Greenberg 1971). Morris (1918) was able to transmit anthrax, Bacillus anthracis, from infected guinea pigs and sheep to healthy guinea pigs in the laboratory. The flies pick up the infection only in the latter stages of the infection; and in nature the flies do not feed on anthrax carcases. Greenberg (1971) cited from two obscure reports only 2 other pathogenic organisms found associated with the horn fly, polio-virus and Trypanosoma congolense, but concluded the fly plays no role in their transmission.

Control

The earliest control measures were nonchemical in nature. Scattering of the manure was recommended to rapidly dry it out and thus render it unsuitable for larval development. Though an effective control measure, it was a very time-consuming operation and therefore not often implemented by the farmer. Bruce (1940) developed a fly trap through which infested animals were forced to pass. When strategically placed, the traps offered relatively effective control of adult flies.

Later, repellents in the form of various oils and grease formulations were employed but were never very effective.

Control of horn flies appeared to be well in hand with the advent of chemical pesticides, as a large number of

materials were shown and continue to be effective if applied on a regular basis. Much research has been directed at developing time- and labor-saving methods of insecticide application. Among these include self-applicating devices such as back and face rubbers, back oilers, and dust bags; insecticide treated collars, blocks, and ear tags are also effective (Rogoff and Moxon 1952; Lindquist and Hoffman 1954). Insecticides and insect growth regulators as feed additives are successful at controlling the larval stages in the manure. However, the main disadvantage of chemical control is the potential contamination of the commodity.

A number of insect species are known to prey upon or parasitize the horn fly. The majority occur in the order Hymenoptera and are pupal parasites (Greer 1975, Escher 1977). Depner (1968) concluded, however, that hymenopterous parasites as they occur in natural situations are not effective in maintaining horn fly populations below economic levels.

Pheromones of the Muscidae

The House Fly

Rogoff et al. (1964) first indicated the presence of a house fly sex pheromone by demonstrating the attraction of males to females in an olfactometer and to fly models (pseudoflies) treated with benzene extracts of female flies. Presence of an olfactory attractant was further confirmed by

Mayer and Thaggard (1966). Subsequently, a number of investigators (Mayer and James 1971; Silhacek et al. 1972a, 1972b) reached various, but incomplete, stages of purification of the attractant; all agreed, however, that the active material was located in the non-polar lipid fraction. Finally, Carlson et al. (1971) successfully identified Z-9-tricosene, or muscalure, as the sex attractant pheromone. Muscalure was later demonstrated to be a mating stimulant as well (Rogoff et al. 1973).

Further studies revealed that Z-9-tricosene was not highly specific and in some instances was not solely responsible for eliciting sexual activity in male flies. Carlson et al. (1973) tested a series of structural analogs of muscalure and found a number to be active, some of which were equal to or slightly exceeded the activity of muscalure. In similar findings, Mansingh et al. (1972) reported attractant activity for the C_{19} to C_{25} series of Z-9-alkene homologs; also, greater activity was shown with a 7:3 ratio of Z-9-tricosene and Z-9-heneicosene than with Z-9-tricosene alone. Uebel et al. (1976) demonstrated an enhancement of mating strike activity by combining with Z-9-tricosene certain methyl-branched paraffins of female origin. Carlson et al. (1973) concluded that perhaps this lower specificity may be characteristic of sex pheromones that are not highly potent.

Other Muscidae

The successful search for the house fly sex pheromone prompted investigation of other muscoid flies. All muscoids studied to date have hydrocarbons as the attractant/mating-stimulant compounds, and most of the more active ones are olefins.

Chaudhury et al. (1972) reported a volatile sex pheromone extracted from mature female face flies (Musca autumnalis De Geer); attraction and mating stimulation of male flies was demonstrated. Chemical analysis suggested the pheromone to be an unsaturated hydrocarbon. Uebel et al. (1975a) successfully isolated the active components of the pheromone which were straight-chain monoalkenes Z-14-nonacosene, Z-13-nonacosene, and Z-13-heptacosene. These compounds were present in both sexes, but the higher proportion of nonacosane and heptacosane in mature males was responsible for weakening the active alkenes.

Investigating the stable fly, Stomoxys calcitrans (L.) Muhammed et al. (1975) reported that the polyolefin fraction of female body hydrocarbons was attractive to mature males while the trans and cis olefins stimulated the males to copulate. Uebel et al. (1975b) found that cuticular extracts of the female stable fly contained saturated and unsaturated hydrocarbons which induced copulation in male flies. The active saturates included mono- and dimethyl-substituted hentria- and tritriacontanes; active unsaturates included Z-9-hentriacontene, Z-9-triatriacontene, and methyl-

branched hentria- and tritriacontenes.

In the little house fly, Fannia canicularis (L.), Uebel et al. (1975a) identified Z-9-pentacosene as a male mating stimulant. Female mono-olefins with 31 and 33 carbons were the most active mating stimulant compounds in F. pusio (Wiedemann) (Uebel et al. 1977a) and the most active of the C_{31} and C_{33} compounds synthesized was Z-11-hentriacontene. In yet another species, F. femoralis (Stein), the C_{31} female mono-olefins were most active in generating male copulatory activity; again, the most active synthetic was Z-11-hentriacontene (Uebel et al. 1977b). Addition of female saturated hydrocarbons increased the activity of the C_{31} mono-olefins.

Langley et al. (1975) reported the discovery of what he termed a sex-recognition pheromone in the tsetse fly, Glossina morsitans. The pheromone was contained in the non-polar cuticular lipid fraction of the female and seemed to be attractive to males over only very short distances. These investigators suspected the active component to be a hydrocarbon with a high carbon number (31-38).

Assays of Muscoid Olfactory Response and Mating Stimulation

Olfactometers

Attraction of insects over distance to a treatment source has been assayed most successfully in the laboratory with specifically designed olfactometers. Design of an

olfactometer for the specific behavior of the target insect is crucial to the success of a bioassay. Beroza and Jacobson (1963) pointed out that the bioassay must be built around the natural habits and idiosyncrosies of a species before valid results can be obtained.

Over the years, olfactometer systems have become more refined, consequently producing more sensitive assays. The refinement has been accomplished by regulating such factors as air velocity, humidity, temperature, sound, and light, and by eliminating contaminating odors. Olfactometer designs have also been altered to facilitate proper cleaning of the apparatus. Ripley and Hepburn (1929) and Schwertfeger (1957) designed olfactometers housing several testing units; speed of obtaining results was therefore increased by permitting multiple replications at a single time. Rapid testing is important when assaying muscoid pheromones, as the innately large variation in response requires extensive replication in order to generate statistically sound data.

The most popular olfactometer designs employ the Y-tube principle, which was first introduced by Barrows (1907). In this design, air passed over test substances is directed through the arms of the Y in the direction of the stem; insects passing through the stem in the opposite direction are presented a choice of entering one of the 2 arms. Based on the numbers entering each arm, the degree of attractiveness of the materials is determined.

Carlson et al. (1971, 1973), Mayer and James (1971), and Silhacek et al. (1972a, 1972b), successfully assayed house fly extracts and related compounds in an olfactometer designed and constructed by Schreck et al. (1967). This "Y-tube" olfactometer consisted of 2 ports in the front face of a large Plexiglas cage (90 x 45 x 54 cm). A glass cylindrical trap was inserted in each port; filter papers treated either with test materials or a standard were positioned individually in the outer ends of the traps. Filtered, humidified outside air was delivered to the front of the olfactometer and then passed sequentially over the samples, through the traps, and into the cage housing the test insects before exiting through the screened rear face of the cage. Counts were made of flies entering the traps in a given time. Mansingh et al. (1972) employed a similar olfactometer but with a much smaller Plexiglas cage (15 x 15 x 50 cm).

Chaudhury et al. (1972) assayed face fly extracts with two types of olfactometers. One was a classic Y-tube consisting of 2 arms (each approximately 5 cm diameter x 25 cm length) and a base or holding chamber (approximately 10 cm diameter x 20 cm length). The arms were divided into 2 compartments, one in which test extracts were positioned and one which served to trap responding flies. Filtered air was delivered into the arms and sequentially passed over the test extracts, through the traps, into the holding chamber, and then finally exhausted at the base of the

holding chamber. Virgin male flies released into the holding chamber were free to move upwind into either arm where they were trapped. The other olfactometer was an enclosed glass cylinder (10.5 cm diameter x 30 cm length) with 2 glass intake tubes opening into it at the base. Fly models (dead female face flies) were attached at the intake tube openings and filtered air passing over test extracts or standards was delivered through the tubes and into the cylinder. Virgin males were released into the cylinder and response was assayed by recording the number of mating strikes by the test males on the fly models.

Schwertfeger (1957) assayed the response of stable flies to the sebaceous secretions of a number of farm animals. His olfactometer consisted of four vertically-oriented glass Y-tubes, eight stainless steel bait jars (connected to the arms of the Y-tubes), a manifold of stainless steel tubing, and a system of steel tubing to convey the air. Materials to be tested were placed in the bait jars, the test insects were placed in the base of each Y-tube and response was assayed by counting the number of flies entering the arms of the Y-tubes. Temperature control was accomplished by immersing the entire apparatus in a large water bath provided with heaters and a thermostat. Humidity was adjusted by passing filtered air through jugs containing given saturated salt solutions. Air delivered to the olfactometer was manipulated so that it passed through each Y-tube arm at an equal and constant velocity.

Muhammed et al. (1975) employed 4- and 2-port vertical olfactometers to test the response of stable flies to whole body fly extracts. Filtered outside air at near constant temperature, humidity, and velocity was delivered through a set of 4 or 2 Plexiglass cylindrical choice chambers (5 cm diameter x 23 cm length) which opened into a large cylindrical mixing-holding chamber (10 cm diameter x 46 cm length). Air exited at the screened base of the mixing-holding chamber and was exhausted to the outside. Test procedures were essentially as described before in the case of Carlson et al. (1971, 1973). Treatments or standards were placed (actually suspended) in their assigned choice chamber(s), test insects moving upward from the holding chamber were then permitted to make a choice by entering the preferred chamber.

Another approach employed by Muhammed et al. (1975) involved a major modification of the Y-tube principle. In this case, the holding chamber common to the 2 or 4 choice ports was replaced with respective sets of 2 or 4 holding chambers of the same size as the choice chambers. Thus, each choice chamber opened into an individual holding chamber containing an allotment of test insects. Upward migration (attraction) into the choice chamber and mating activity were compared among the 2 or 4 separate units. In a similar approach, Daykin and Kellogg (1965) developed an olfactometer which separated treated and untreated air streams so as to eliminate cross-contamination.

Response of horn flies in an olfactometer was studied by two groups of investigators. Kinzer et al. (1970) tested human emanations and cow odor in a device patterned after Daykin and Kellogg (1965). Flies were allowed to move upwind in 2 glass tubes which separately housed control and treatment air streams. Responding flies were trapped in chambers at the ends of the glass tubes. Air entering the olfactometer was filtered and preconditioned to a given temperature and RH. Flowmeters verified balance of flow between the air streams. The light effect was minimized by uniformly illuminating the test chambers with fluorescent "red light". Response, however, was minimal, and positive treatment - control ratios ranged from only 1.57:1 to 2.40:1. Hargett and Goulding (1962) failed in their efforts to assay olfactory responses of horn flies in a Y-tube olfactometer; however, they were able to report a lack of humidity preference on the part of horn flies based on Y-tube trials. In each case no description of the olfactometer was given.

"Pseudofly" Tests

By far the most popular assay for chemical mating stimulation of muscoid flies is the "pseudofly" test. The test was first introduced by Rogoff et al. (1964, 1973) and subsequently numerous variations of the technique have seen devised as research of muscoid pheromones has expanded.

Pseudofly tests are designed to induce the fly species in question to perform its mating ritual under artificial conditions. In the cases of the house fly (Rogoff et al. 1964, 1973; Carlson et al. 1973; Uebel et al. 1976), the little house fly (Uebel et al. 1975a), the stable fly (Muhammed et al. 1975), Fannia femoralis (Uebel et al. 1977b), and F. pusio (Uebel et al. 1977a), a fly-sized knot (the pseudofly or fly model) cut from black yarn or shoelace is used to simulate the visual stimuli that elicit mating behavior patterns in the male flies. Uebel et al., (1975b, 1975c) used impaled males of the species as fly models when working with face flies and stable flies. Test chemical extracts or solvent alone impregnated on the knots or male flies act as chemical stimuli and complete the fly model. Mating response is assayed by visually counting and then comparing the number of "strikes" or "jumps" (Murvosh et al. 1964) by male flies on treated and untreated fly models per given unit of time. Muhammed et al. (1975) and Harris et al. (1976) recorded counts in another way: Fly models were connected to phonograph cartridges which were wired through a recording device; contacts between test flies and fly models triggered the phonograph cartridges and were automatically recorded.

Pseudoflies and test males were confined in petri dishes (Rogoff et al. 1964, 1973; Carlson et al. 1973) or quart jars (Uebel et al. 1975a, 1975b, 1975c, 1976, 1977a, 1977b). Working with the tsetse fly, Glossina morsitans, Langley

et al. (1975) assayed the response of male flies to variously treated dead flies in a glass tube. The density of test insects apparently has little influence on success of the assay.

Field Trials

To date, field evaluation of muscoid pheromones has been extremely limited. In fact, only muscalure, the house fly sex pheromone, has received attention at this stage of testing.

Carlson and Beroza (1973) employed the following series of traps to evaluate the attractancy of muscalure: sticky panels, fly paper strips, sugar bait, electric grids, and several kinds of box traps. Except for the box traps which proved to be ineffective, the addition of muscalure to the remaining traps increased house fly capture from 2.8 to 12.4 times depending on the trap. Male and female flies were trapped in equal numbers in contrast to laboratory olfactometer studies where only males were attracted. Morgan and Gilbert (1973) evaluated various dosages of muscalure used in combination with sugar fly bait; attractancy was found to be directly proportional to dosage. Again, both males and females were equally attracted.

Muscalure is commercially available, most commonly in combination with sugar fly baits.

METHODS AND MATERIALS

Biological Material

Sources

Adult horn flies were the sole source of test insects and fly lipid extracts. Insects and lipid extracts for bioassay purposes were obtained from a laboratory colony established at the Department of Entomology and Nematology, University of Florida (hereafter FLS - Florida Laboratory Strain) (Greer 1975).

Horn fly lipid extracts for the purpose of chemical analysis were obtained from 3 sources: (1) the above Florida Laboratory Strain (FLS), (2) a laboratory colony established at the USDA Livestock Insects Laboratory, Kerrville, Texas (KLS - Kerrville Laboratory Strain), and (3) laboratory reared F_1 generation of wild horn flies captured at a pasture near the University of Florida campus (FWS - Florida Wild Strain). Fecal lipid samples of the FLS were also the subject of chemical analysis.

Adult house flies were reared for analysis and comparison of paraffins. These flies were the Orlando Regular strain and were obtained from the USDA, Insects Affecting Man Laboratory, Gainesville, Florida.

Rearing of Horn Flies

Pre-imaginal maintenance of the FLS was essentially as described by Greer (1975). Adults of the FWS were allowed to oviposit in the laboratory, and the pre-imaginal forms were reared in the same manner as the FLS. No data were obtained on pre-imaginal maintenance of the KLS.

Adults of all 3 strains were maintained identically. The flies were housed in aluminum-framed cages (25.5 x 24 x 15 cm) covered with 2 layers of tube gauze (Tubegauz^{R}, The Scholl Mfg. Co., Inc., Chicago, Ill.) and were provided once daily with bovine blood on cotton balls. Blood was treated with antibiotics (0.2g kanamycin and 30,000 units mycostatin per liter) and an anticoagulant (0.4g sodium citrate per liter). Temperature was maintained at 27 ± 1.1^{o}C, relative humidity at 50 ± 10%, and lighting was continuous.

Adults were either reared together as mixed-sexes (considered mated) or sexed within 24 hours of eclosion and reared separately (considered virgin). Sexing was facilitated by immobilizing the flies in a cold room (1^{o}C). Groups of flies were reared to a desired age and were either frozen (-20^{o}C) and then extracted when sufficient numbers accumulated, or used as bioassay test insects.

Chemistry

Extraction of Crude Lipids

Whole body extractions

Horn flies. A given sample of frozen flies were immersed in hexane (at the rate of 1000 flies/50 ml hexane)

and allowed to stand overnight; the following day the extract was decanted through filter paper (Whatman No. 1). The flies were then ground in a mortar, re-immersed in fresh hexane overnight, and the extract once again filtered the following day. The two collections of extract were combined and the solvent was evaporated under vacuum with a rotary evaporator; all remaining traces of solvent were removed by evaporation under a stream of N_2.

Whole body extracts were made of the following groups of flies: (1) 3-4 day old mixed-sex FLS, (2) 3-4 day old virgin males FLS, (3) 3-4 day old virgin females FLS, (4) under-24-hour-old males FLS, (5) under-24-hour-old females FLS, (6) 3-4 day old mixed-sex KLS, (7) 3-4 day old virgin males FWS, and (8) 3-4 day-old virgin females FWS. All groups were subjected to chemical analysis of the hydrocarbon fraction; groups 1-3 were sources of test materials for bio-assays.

House flies. Four-5 day old, separately reared (virgin), male and female house flies were extracted for cuticular lipids. Frozen flies were immersed in 50 ml of petroleum ether for 3 hours, the solution occasionally agitated, and then the extract filtered. The flies were then placed in a glass funnel fitted with filter paper and washed once again with 50 ml of petroleum ether. The extracts were combined and solvent removed as previously described. Chemical analyses were made of the paraffin fraction of the hydrocarbons.

Fecal extractions

A sample of fecal lipids of the FLS were obtained from a colony cage heavily contaminated with fecal material. All flies were removed and the cage was washed down with 300-400 ml of hexane dispensed with a squeeze bottle; the cloth cage sleeves were soaked in hexane for several minutes then squeezed by hand to remove excess solvent. The cage and the sleeve washes were collected and filtered; the solvent was removed in the same manner as with the whole body extracts. The hydrocarbon fraction of the crude lipid extract was subjected to chemical analysis; fecal lipid extract itself was not bioassayed.

Analysis

Fractionation of major lipid classes

Crude lipid extracts were fractionated on 2 x 45 cm columns of silica gel (60-200 mesh, J. T. Baker Chemical Co., Phillipsburg, N. J.)(Silica gel-LC). The hydrocarbons (saturates and unsaturates) were eluted with 200 ml of n-hexane (Phillips Petroleum Co., washed with conc. H_2SO_4 2 days, washed with water, then distilled from metallic sodium), the polyunsaturates (polyolefins) with 200 ml of 1% ether in hexane, and the remaining more polar lipids with 200 ml each of 5%, 10%, and 50% ether in hexane. Separation of hydrocarbons from the other major lipid classes was confirmed by thin layer chromatography (TLC) on silica gel plates (250 μ Anasil, Analabs, Inc., North Haven, Conn.) using hexane:

ether:acetic acid (90:10:1) solvent.

The hydrocarbons and polyolefins were the only lipid classes analysed further. Initial olfactometer bioassays showed activity to be associated with the hydrocarbons, while the lipids eluted with solutions of ether in hexane showed no activity. All other muscoid flies studied to date, except for the stable fly, exhibit this same pattern of activity. The polyolefins were examined because this fraction in the stable fly had been demonstrated to be an attractant (Muhammed et al. 1975).

The hydrocarbons were further separated into paraffins (saturates) and mono-olefins (unsaturates) on 1.3 x 36 cm columns, packed 20% silver nitrate ($AgNO_3$) impregnated silica gel (60-200 mesh, Hi-Flosil-Ag, Applied Science Laboratories, Inc., State College, Penn.) ($AgNO_3$-LC). The paraffins were eluted with 60 ml of hexane and the mono-olefins with 100 ml of 2% ether in hexane. Separation and purity were confirmed by TLC on silica gel plates impregnated with 20% silver nitrate (150μ Uniplates, Analtech Inc, Newark, Del.) with paraffin and cis and trans mono-olefin standards ($AgNO_3$-TLC). The solvents were removed under vacuum with a rotary evaporator and under a stream of N_2.

Initial characterization of hydrocarbons

The paraffins and/or mono-olefins were partially characterized by analytical gas chromatography (GC) on a Varian Model 2100 with glass columns (1.8 m x 2 mm ID) packed with

3 or 5% SE-30 on 120-140 mesh Gas Chrom Q, and flame ionization detection (FID). Polyolefins (1% ether in hexane eluents) from 3-4 day old mixed-sex FLS were also characterized. The major n-paraffins and chain lengths of branched paraffins were determined by co-injection of known n-paraffin standards. Chain lengths of the major mono-olefins were likewise determined and Kovats' indices (KI) (1966) assigned.

Identification

Paraffins. Electron impact mass spectra (EI/MS) were obtained for branched paraffins from extracts of male and female FLS flies (3-4 days old, virgin). The EI/MS were obtained with a Varian Mat CH5 mass spectrometer interfaced via a membrane separator to a Varian 1400 GC which was equipped with 3.2 m x 2 mm ID glass columns of 3% OV-1 on 100-200 mesh Gas Chrom Q.

Prior to analysis, the paraffin samples in iso-octane solutions were subjected to a 5A molecular sieve after the methods of O'Connor et al. (1962). The molecular sieve preferentially absorbed the n-paraffins and removed the majority of them from solution; elimination of the n-paraffins facilitated the analysis of the branched paraffins.

Mono-olefins. The mono-olefin fraction from all sources of horn flies consisted of 4 major peaks which eluted at KI 2272 and 2292 (C_{23}), KI 2477 (C_{25}), and KI 2677 (C_{27}), and are hereafter designated I, II, III, and IV, respectively.

Each was collected from a FLS female (3-4 days old, virgin) mono-olefin sample by preparative GC on a Varian Model 90-P Aerograph; the C_{23} compounds were collected off a stainless steel column (6 m x 4 mm ID) packed with 5% SE-30 on 100-200 mesh Gas Chrom Q, thermal conductivity (TC) detection, and the C_{25} and C_{27} compounds collected off a stainless steel column (3 m x 2 mm ID) packed with 3% SE-30 on 100-200 mesh Gas Chrom Q, TC detection.

The individually trapped compounds were ozonized to determine sites of unsaturation and the resulting aldehyde fragments identified by GC analysis after the method of Beroza and Bierl (1967). The ozonides of I and II were identified on 3 gas chromatographs equipped with 3 different columns: (1) F and M Model 810 with a stainless steel column (3 m x 2.2 mm ID) packed with %5 Carbowax 20 M on 100-200 mesh Gas Chrom Q, FID, (2) Varian Aerograph Series 1200 with a stainless steel column (2 m) packed with 5% HI-EFF-1BP on 80-100 mesh Chromosorb W AW, FID, and (3) the previously described Varian Model 2100 with the 5% SE-30 glass column. The short chain aldehyde obtained from ozonolysis of II could not be detected on the above columns; instead, attempts were made to detect it on a stainless steel column (3 m x 2.2 mm ID) packed with 80-100 mesh Poropak Q and a glass column (1.8 m x 4 mm) packed with 50-80 mesh Poropak Q. The ozonolysis products of III and IV were identified only on the Varian Model 2100 gas chromatograph with the previously described 5% SE-30 glass column.

I and II were hydrogenated at atmospheric pressure over neutral palladium on charcoal catalyst to determine presence of branching by GC analysis. III and IV were not hydrogenated. EI/MS were obtained for the hydrogenated II, but not for the hydrogenated I. Infrared spectra of II to determine presence of a vinyl double bond were obtained on a Perkin Elmer Model 221G in solutions of CCl_4 and as films on sodium chloride plates.

Chemical ionization mass spectra (CI/MS) of the ozonides of the 4 respective mono-olefins and of II parent material were obtained with a Finnigan 1015C mass spectrometer, interfaced to a Systems Industries 150 data system. A Varian Model 1400 GC, equipped with either a 1.8 x 2 mm ID glass column packed with 3% OV-1 on Gas Chrom Q (100-120 mesh) or a 1.8 m x 2 mm ID stainless steel column packed with 5% SE-30 on Gas Chrom Q (100-120 mesh), was used as the inlet. Methane, used as the carrier and ionizing gas, was passed directly into the ion source where pressure was maintained at 1.0 torr, and the GC oven was temperature programmed. The computer data system provided mass spectra, reconstructed gas chromatograms (RGC), and limited mass range searches (LMS), representing ions of specific masses plotted versus spectrum number.

Synthesis

The material which eluted from an SE-30 column at KI 2292 (II), which was identified as Z-5-tricosene, was

synthesized for this study. Synthetics of the remaining 3 mono-olefins (I, III, and IV) were obtained from Dr. Dave Carlson, USDA, Insects Affecting Man Laboratory, Gainesville, Fla. (I), and Dr. E. C. Uebel, USDA Chemical and Biophysical Control Laboratory, Beltsville, Md. (III and IV).

Z-5-tricosene was synthesized by Dr. Dave Carlson. It was prepared by the Wittig reaction as follows. 1-Bromo-octadecane (Aldrich, 52g, 0.156 mole) and 52g (0.2 mole) of triphenylphosphine (Aldrich) were dissolved in 200 ml of acetonitrile and refluxed overnight in N_2 atmosphere. The acetonitrile was distilled off on a rotary evaporator, and the residue was poured into anhydrous ether. The white solid that precipitated upon stirring was collected and dried thoroughly under vacuum to give octadecyltriphenyl-phosphonium bromide.

Octadecyltriphenylphosphonium bromide (34g, 0.057 mole) was dissolved in 100 ml of anhydrous tetrahydrofuran (THF) in a dry flask under N_2. The solution was stirred, cooled in an ice bath, and held between 10 and 20°C, while 30 ml of butyllithium (15.16% solution in hexane, Foote Mineral Co.) was added slowly. The dark red solution was held at 15-20°C for 1 hour and then cooled to 10°C. Freshly distilled pentanal (5.6g, 0.065 mole, bp 102-103°C (16mm)) was added dropwise with stirring; the solution was allowed to warm to room temperature overnight. Two days later the reaction mixture was diluted with water and hexane, shaken, and the organic layer separated. The mixture was washed one more

time with water, then with a sodium chloride solution, and then dried over sodium sulfate. Rotary evaportation gave 25g of crude oil and a few crystals of triphenylphosphine oxide. The crude oil was diluted with hexane and portions were passed 3 times through 2 x 50 cm columns of silica gel (60-200 mesh, Baker) with 200 ml hexane each time. The eluents were combined and removal of solvents gave 13.5g (74%) of olefin estimated to contain 85% cis isomer and 15% trans isomer of 5-tricosene. The cis and trans isomers from a 2g sample of the olefin were separated by $AgNO_3$-LC; the trans isomer was eluted with 120 ml of hexane and the cis isomer with 150 ml of 1% ether in hexane. Separation and purity of the isomers were confirmed by $AgNO_3$-TLC.

Quantitation of hydrocarbons

In some cases, the isolated, solvent-free crude paraffins and crude mono-olefins were weighed on a Mettler Balance (Mettler Instrument Corp., Hightstown, N.J.). Quantitations of the paraffins and major mono-olefins were obtained by GC by comparing peak deflections or peak areas with standards of known concentration; peak areas were measured on a Hewlett-Packard Model 3380A Integrator.

Bioassays

Two methods were employed to assay the activity of natural (extracted) and synthetic horn fly body lipids. The assays were designed to cover the spectrum of pheromonal

responses seen in other muscoid flies. Although both methods have received extensive use previously, major modifications were necessary in order to fit the specific behavior nuances of the horn fly.

Two olfactometers were utilized to measure attraction of horn flies to a chemical source over distance. One was a modification of the 4-port olfactometer developed by Muhammed et al. (1975); the other was a newly developed four-unit olfactometer. A variation of the pseudofly test (Rogoff et al. 1964, 1973) was devised in an attempt to measure short-range attraction and contact stimulation.

Modified Four-Port Olfactometer

The 4-port olfactometer of Muhammed et al. (1975, Fig. 1.) received only limited use. It was altered by closing off 2 of the ports, thus leaving only 2 choice chambers functional, one as treatment and one as check. The altered design more closely resembled a simple Y-tube. Operating temperatures were increased to the preferred range of the horn fly, 32.5 $\pm$ 1.5^{o}C. Otherwise, the olfactometer design and test methods remained the same.

Four-Unit Olfactometer

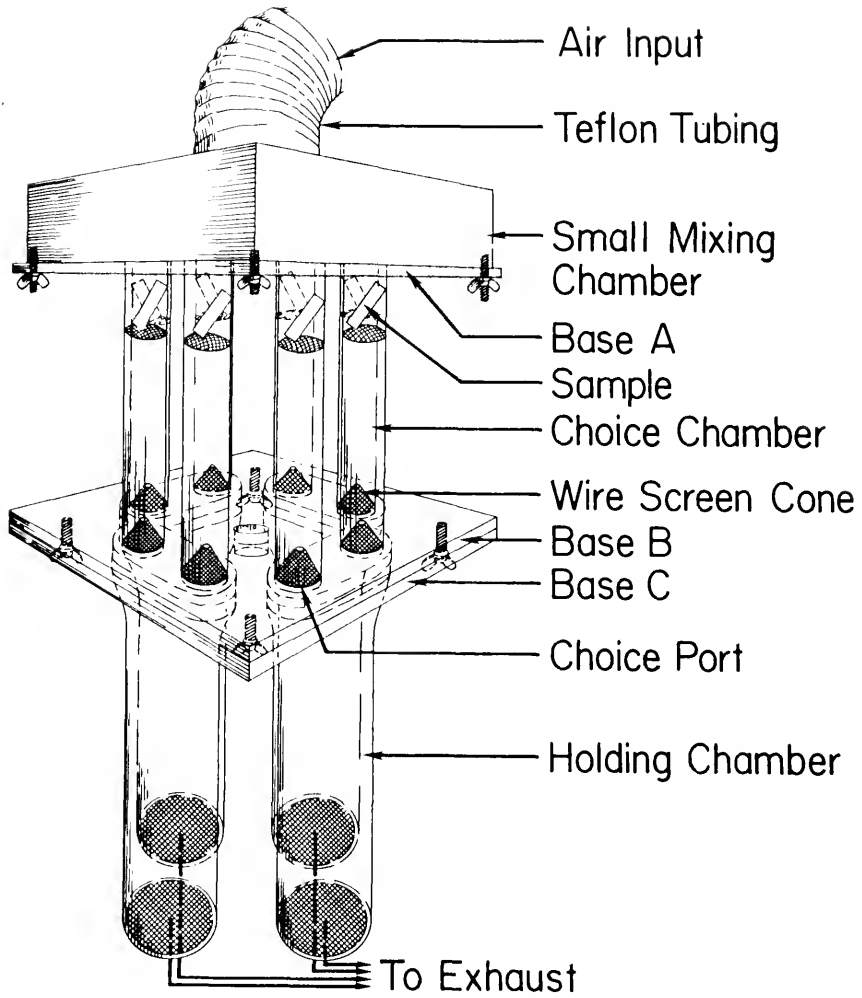
The four-unit olfactometer was based on the classic "Y" tube principle. In the description of the olfactometer proper, the arms of the Y are choice chambers and the stem is the holding chamber. The design was modified such that

4 replications could be run almost simultaneously.

Design

Olfactometer proper. A diagramatic illustration of the 4-unit olfactometer is presented in Fig 1. A battery of 8 vertically oriented glass choice chambers (each 30 cm length, 3.5 cm ID, 4.5 cm OD) were equally spaced (2 cm apart) in a circle (15 cm diameter) and secured by 2 Plexiglass^{R} bases (23.5 x 22.5 x 1.2 cm; base A above, base B below). The chambers opened at each end through holes cut in the bases. An additional base (C) of the same dimensions was bolted to base B; 4 oblong holes (10.2 x 4.5 cm) cut in this base were arranged so that the 8 choice chambers opened to the bottom in 4 sets of 2. Four Plexiglas holding chambers (30 cm length x 7.0 cm ID, 7.5 cm OD) were heat molded at one end such that they were able to fit and seal the oblong openings of the 2-port sets. The holding chambers were closed at the opposite end with wire screen. Thus in each of the 4 sets, 2 choice chambers opened at the bottom into a Plexiglas holding chamber; test insects moving upward from the holding chambers were therefore offered a choice of 2 ports to enter, i.e., treatment or check. Inverted wire screen cones were implaced in each port; the apex of each cone had an 8 mm diameter hole through which test insects passed on entering a choice chamber. The cone and a wire screen barrier placed approximately 3/4 the way up the length of a choice chamber served to confine the responding insects. Size of choice

Figure 1. The four-unit olfactometer.


Air Input
Teflon Tubing
Small Mixing Chamber
Base A
Sample
Choice Chamber
Wire Screen Cone
Base B
Base C
Choice Port
Holding Chamber
To Exhaust

and holding chambers were selected to minimize changes in air flow between the two types of chambers.

Initially, a fluorescent lamp (hereafter referred to as PL; F40PL - Plant Light^{R} Fluorescent, General Electric Co.) was used to illuminate the olfactometer. It was later replaced with another fluorescent lamp (Cool White^{R} Fluorescent Lamp; Sears) which was ensheathed in red plastic and thus emitted "red light". The lamp was positioned along the axis about which the choice and holding chambers were arranged (not shown in Fig 1).

Air supply system. An electric squirrel cage fan drew outside air through a length of 5 cm PVC pipe and into one end of a large Plexiglas mixing chamber (93 x 54.5 x 37 cm). The chamber contained a water bath from which a gauze sail extended and was heated by a Fenwal^{R} regulated hot aluminum plate. Incoming air was directed on the sail, was further mixed by obstacles of loosely wadded aluminum foil, and exited at the opposite end of the chamber. The heated, humidified air then passed via a length of 10 cm diameter Teflon^{R} tubing to a small, Plexiglas mixing chamber (21 x 21 x 9.5 cm) mounted over and secured to base A of the olfactometer proper (Fig 1). An activated charcoal filter to remove contaminating odors, a heating element, and a temperature sensor were located in the teflon tubing near its junction with the small mixing chamber. The heating element and the temperature sensor were connected with a proportional temperature controller (RFL Model 879, RFL

Industries, Boonton, New Jersey), maintaining the passing air at a constant regulated temperature. Dry and wet bulb thermistor probes in the small mixing chamber monitored the temperature and relative humidity of the passing air; probes were connected through a switch box and a calibrated strip chart recorder. Velocity of air exiting the choice chambers was measured by an anemomenter (Alnor^{R} Type 8500 Thermo-Anemometer, Alnor Instrument Co, Chicago, Illinois) once per day before bioassays were conducted. A baffle in the large mixing chamber consisting of a small hole with a sliding door was used to adjust air speed. Thus, air entering the small mixing chamber passed down through the choice and holding chambers and exited at the screened bases of the holding chambers. A white smoke was introduced to the small mixing chamber to evaluate the air flow and mixing through the choice and holding chambers (Fig 2); the smoke was generated by exposure of a small sample of titanium tetrachloride to the moist air. A small platform (120 x 47 cm) situated 25 cm below the holding chamber bases was placed over an exhaust fan which drew the exiting olfactometer air to the outside.

Air temperatures were maintained in the preferred range of horn flies, 32.5 $\pm$ 1.5^{o}C. The fine adjustment on the temperature controller was sensitive enough to maintain only within $\pm$ 1.5^{o}C. Humidity was less easily controlled, despite specific design attempts to maintain a high, uniform humidity; relative humidity was maintained at 60$\pm$10%. However,

Figure 2. Air-flow through choice and holding chambers of the four-unit olfactometer.

Harget and Goulding (1962) found no difference when comparing the response of horn flies to "wet" and "dry" air in a Y-tube olfactometer.

Adjustments for lighting effects

Lighting significantly affected horn fly responses. Horn flies are strongly phototactic (Harget and Goulding 1962; Bruce 1964; Morgan 1966), so balance of lighting was essential to prevent masking of olfactory responses. Considerable time, therefore, was spent in developing olfactometer light balance by assaying the balance of response among all choice chambers and between choice chambers comprising a set. Adjustments for unequal fly responses included altering the vertical position of the PL and employing holding chambers of 2 different lengths (30 cm vs 25 cm). Altering positions of the choice chambers was not possible because they were fixed as a unit. Effects of adjustments on fly response were monitored in tests where flies were allowed to respond to "double blanks."

Developmental changes in the position of the PL affected movement of horn flies in 2 ways: (1) the rate at which flies move out of the holding chambers and upward into the choice chambers (changes in vertical position of the lamp), and (2) movement into choice chambers more illuminated than others (any position off the axis, consequently some choice chambers became preferred over others). The longer of the 2 holding chambers (30 cm) rendered a slightly slower rate

of upward movement by flies. Adjustments were finalized when imbalances among choice chambers were minimized as much as possible and when the desired rate of upward movement by flies was achieved. These final adjustments included (1) the vertical positioning of the PL (41.5 cm length) such that it extended 13 cm above (illuminatingthe choice chambers) and 26 cm below (illuminating the holding chambers) contiguous bases B and C, (2) centrally locating the lamp such that it extended along the axis about which the choice and holding chambers were arranged, and (3) selection of the 30 cm holding chamber. As time went on, however, imbalances became re-established and it became evident that the light variable had to be eliminated as much as possible rather than only balanced. Accordingly, the PL was replaced with the Sears Cool White^{R} Fluorescent Lamp ensheathed in red plastic to produce the desired "red light" (hereafter referred to as RL); assays of horn fly visual acuity conducted by Drs. Herndon Agee and John Davis (pers. comm., Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA, Gainesville, Fla.) show the red region of the visible electromagnetic spectrum to generate the least response. Tests under red light conditions were conducted with room lights off. Red light impingement on the choice chambers ranged from 70 to 85 lumens/m^2.

Testing methods

All test insects were 3-4 days old (considered sexually mature, Harris et al. 1968) and virgin. Males and females

were test separately. On the night before testing, the insects were immobilized in a cold room (1°C) and transferred in lots of 40 or 20 to plastic cups covered with serex. A small fly-feeding pad of blood-saturated cellucotton was placed on the serex covers, it was changed the following morning, and finally removed about one hour before testing the flies in the olfactometer.

Test materials in hexane solution, ranging from 6-50 µl, were uniformly deposited by a syringe over half of one side of a glass microscope slide. Check slides were likewise treated but with approximately 25 µl of hexane only. Four replications of 1 treatment and 1 check slide each were then suspended from or supported above the wire screen barriers in respective choice chambers of the olfactometer and the inverted wire screen cones implaced at the bases. Thus, 4 separate tests could be run almost simultaneously. To correct for imbalances due to light and position variables, any given choice chamber served as a "treatment" and "check" an equal number of times when a given material was tested.

Each test assayed the response 40 test flies (when the PL was employed) or 20 test flies (when the RL was employed) to the test material and hexane check. Test flies were anesthetized by CO_2 for transfer to a holding chamber which was fitted to 2 adjacent choice chambers. This process was repeated for the remaining 3 sets. Recovery time was not considered; a particular test started when the holding chamber was secured in place. Counts were made at the end

of 30 minutes exposure; flies entering a choice chamber or congregating within the confines of the screen cone at the base were considered as responding to the chamber. At the end of each set of tests, flies were discarded and the choice and holding chambers were disassembled and thoroughly washed with hot water and detergent.

Test Materials for Olfactometer Assays

Female and male crude lipid extracts were assayed. Total hydrocarbons, mono-olefins, and paraffins for assay purposes were recovered from mixed-sex rather than virgin flies, as the male and female GC profiles for these respective groups of compounds were very similar (Fig 4, 6, and 8). Because of limited material, the synthetic counterparts of the individual mono-olefins were assayed in lieu of the natural material. A flow chart (Fig 3) illustrates the route followed for assaying the active materials.

Determination of Dosage Rates for Olfactometer Assays

In an olfactometer, only that portion of a test material which enters the air stream is available to affect the test insects. Thus, the amount of a test material applied to a glass slide does not necessarily reflect the dosage to which test insects are subjected. In an effort to determine the approximate amounts of test materials entering the air stream, a study was undertaken to measure evaporation rates of muscalure (Z-9-tricosene) in the 4-test-unit olfacto-

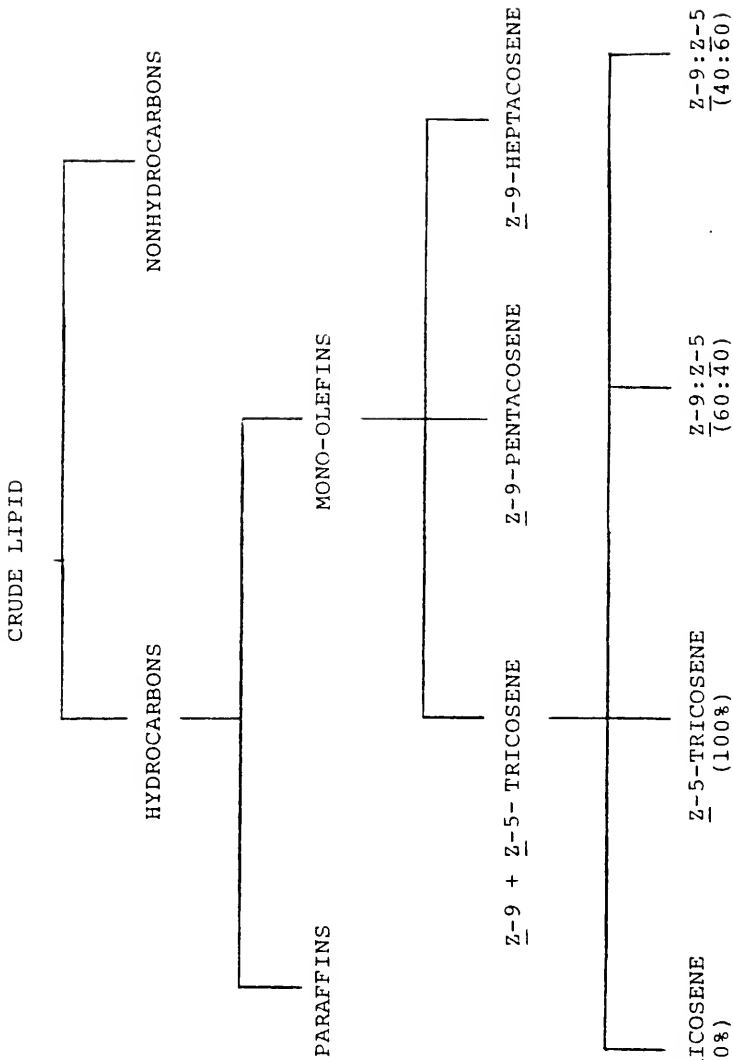


Figure 3. Flow chart illustrating the course of bioassay tests.

meter. Muscalure was chosen because of its chemical similarity to compounds found in active mono-olefin fractions, and because of its availability.

Slide treatment and operation of the olfactometer were exactly as described with the bioassay trials. Losses from 100 ug and 1 mg deposits on glass slides were monitored over 72 hours. At intervals of 0, 12, 24, 48 and 72 hours, a given set of treated glass slides were individually washed with 10 ml of hexane to remove the unevaporated muscalure. Each wash was collected and reduced to 1 ml under a stream of N_2. The muscalure contained in each wash was quantitated on GC in the same manner as previously described for the paraffins and major mono-olefins. Four replicates for each concentration and each time interval were made. A regression analysis of muscalure loss on exposure time was performed.

Test for Contact Stimulation

The design for the contact stimulant test was based on the conventional pseudofly technique (Rogoff et al. 1964, 1973). As was previously discussed, all reported assays of muscoid mating stimulants were variations of this test model.

In addition to mating stimulation, the pseudofly test is described as assaying other chemically based behavioral responses. These include short range attraction (i.e. over a few centimeters) (Rogoff et al. 1964) and sex-recognition (Langley et al. 1975); there may possibly be others. No

attempt was made to distinguish among these responses; all were placed under the one category of "contact stimulation" but were considered to relate to courtship behavior.

In establishing a pseudofly assay, it is first necessary to produce a set of conditions in which male flies will strike a female fly. These conditions then must be maintained when the female fly is replaced with an object treated with test material. For a valid test, it is essential that male flies be induced to strike this object when it is treated with "biologically active" materials. In assays with other muscoid flies, the object replacing the female fly has been either a male fly or a pseudofly (a fly-size knot of black yarn or shoelace).

Preliminary attempts at establishing a pseudofly assay were unsuccessful. Various numbers of mature, mixed-sex, virgin horn flies were confined in battery jars, mason quart jars, and petri dishes for extended periods of time but with no mating activity observed. Flies exhibited exploratory and/or escape types of behavior, i.e., they would constantly search and probe the container in which they were confined; male-female contacts were frequent but appeared to be entirely due to chance and never was a sexually-based response observed. In tests employing petri dishes, house and stable flies (2 males and 2 females in each case) used as controls readily exhibited the characteristic courtship behavior as described by other investigators (Murvosh et al. 1964; Tobin and Stoffolano 1973a; Colwell

and Shorey 1975; Muhammed 1975). It was not until large numbers of horn flies were confined in even smaller volumes that mating activity was induced (after Schmidt et al. 1972). Densities found necessary for the inducement of mating activity ranged from 0.5-1.0 flies/cm^3, although other untested densities outside this range may have also been suitable. The factor of density apparently does not influence the success of other muscoid mating assays.

Accordingly, the following assay was established: Fifteen or 25, 3-4 day old virgin male or female horn flies were confined in a 20 ml glass beaker; the beaker was then covered with a piece of white muslin with a small hole in the center and secured with a rubber band; a small cork anchoring an insect pin was mounted in the hole in the muslin cover such that the point of the pin was situated centrally inside the beaker. Response was assayed by counting the number of contacts made by test insects with a treated fly or pseudofly (a fly-size knot of black yarn) impaled on the pin. Only contacts of 3 seconds duration or longer were recorded, thus flies did not necessarily have to assume copulatory stances or display any other specified courtship behavior. Contacts greater than 3 seconds were not considered chance encounters, and untreated controls verified insect response due to treatment. Beakers were placed on their sides and generally 4, 30-minute trials could be observed at any one time. Test flies were sometimes repeatedly used for up to 2 additional tests, however the

treatment was altered for each test. No food was provided during tests, but was made available up to 1 h before testing commenced. Temperature, relative humidity, and incident fluorescent light were maintained at 26.5±1.5°C, 45±10%, and 300 lumens/m^2 respectively.

Response by male test flies to the following treatments was assayed: A live female fly, a live male fly, a dead female fly,a female fly washed with hexane, and dead male flies and/or pseudoflies treated with various female extracts or a single concentration of synthetic mono-olefins. Response of female test flies to a live male fly and a live female fly was compared.

RESULTS

Chemistry

Paraffins

Florida laboratory strain

GC's of the male and female paraffins (3-4 day old virgin flies) are illustrated (Fig. 4). Identification by EI/MS of the individual components is presented in Tables 3 and 4 for males and females, respectively. Identification was possible for major components but not for some minor components. Quantitations of total paraffins and individual components are presented in Tables 1 and 2, respectively.

The bulk of the components were odd-numbered, straight-chain molecules 21 to 29 carbons in length; much smaller amounts of even-numbered, straight-chain molecules 22 to 28 carbons in length and methyl-branched compounds were present. Identified branched paraffins were either mono- or di-methyl with the most common sites of methyl substitution being the 9, 11, 13, and 15 positions. Qualitatively, the chromatographic profiles appeared to be identical for both sexes; however, some quantitative differences existed between co-eluting peaks. Paraffins comprised 35% and 45% of the total hydrocarbons in 3-4 day old males and females,

Figure 4. Analytical GC's of total paraffins recovered from whole body extracts of virgin male (left) and female (right) 3-4 day old FLS horn flies.

2100
2500
2700
2900
3100

2100
2500
2700
2900
3100

Table 1. Quantitation of total paraffins and mono-olefins recovered from horn fly whole body extracts.

Source	Age	µg of Component Recovered/Fly		
		Paraffins	Mono-olefins	Total Hydrocarbons
FLS Males	24 h	6.56	1.09	7.65
	3-4 days	2.81	5.13	7.94
FLS Females	24 h	2.72	0.59	3.31
	3-4 days	5.82	7.17	12.99
FWS Males	3-4 days	3.86	2.12	5.98
FWS Females	3-4 days	5.44	2.34	7.78

Table 2. Quantitation[a] of paraffins recovered from whole body extracts of 3-4 day old, male and female FLS and FWS horn flies.

Kovats' Index[b]	Source			
	Florida Laboratory Strain		Florida Wild Strain	
	Male	Female	Male	Female
2100	19.7	10.5	7.9	1.0
2130	0.2	0	1.9	0.4
2200	1.1	0.9	2.1	0.8
2300	36.3	27.5	11.6	3.1
2330	0.6	0.4	0.4	0.1
2350	0.1	0.1	0.1	0.4
2400	0.6	0.7	1.5	1.7
2500	15.7	18.9	20.0	22.8
2550	0.3	0.2	0.4	0.4
2600	0.6	0.8	2.2	2.9
2700	13.7	22.4	25.6	42.9
2730	0.5	0.7	2.0	2.5
2750	0.2	1.7	3.6	5.3
2800	0.2	0.5	1.7	2.6
2900	4.8	6.4	3.6	5.6
2930	2.0	3.0	0.9	0.9
3100	0.5	0	-	-
3130	1.9	3.5	-	-

[a] - % of total.
[b] - Unbranched (normal) paraffins have KI values of 2100, 2200, 2300, etc. by definition; KI values of 30 (eg, 2130) are mono-methyl branched by EI/MS; KI values of 50 (eg, 2150) are di-methyl branched by EI/MS.

Table 3. Components of the paraffins recovered from whole body extracts of male FLS horn flies.

Kovats' Index	Structure	Observed Fragments m/e	M-15	M
2130	9 - $C_{21}H_{43}(CH_3)$	140, 196	-	-
	11 - $C_{21}H_{43}(CH_3)$	169	-	-
2200	$C_{22}H_{46}$	-	none	310
2300	$C_{23}H_{48}$	-	none	324
2330	9 - $C_{23}H_{47}(CH_3)$	140, 224	none	none
	11 - $C_{23}H_{47}(CH_3)$	168, 196		
2400	$C_{24}H_{50}$	-	none	338
2500	$C_{25}H_{52}$	-	none	none
2530	7 - $C_{25}H_{51}(CH_3)$	113, 281	none	none
	9 - $C_{25}H_{51}(CH_3)$	252, 253 141, 142		
	11 - $C_{25}H_{51}(CH_3)$	168, 169 224, 225		
	13 - $C_{25}H_{51}(CH_3)$	196, 197		
2550	9,13 - $C_{25}H_{50}(CH_3)_2$	141, 197 211, 267	none	none
	11,15 - $C_{25}H_{50}(CH_3)_2$	169, 239		

Table 3. Continued.

Kovats' Index	Structure	Observed Fragments m/e	M-15	M
2600	$C_{26}H_{54}$	-	none	none
2730	7 - $C_{27}H_{55}(CH_3)$	112, 308	379	none
	11 - $C_{27}H_{55}(CH_3)$	169, 253		
	13 - $C_{27}H_{55}(CH_3)$			
2900	$C_{29}H_{60}$	-	none	none
2930	7 - $C_{29}H_{59}(CH_3)$	112, 337	none	none
	9 - $C_{29}H_{59}(CH_3)$	141, 309		
	11 - $C_{29}H_{59}(CH_3)$	168, 281		
	13 - $C_{29}H_{59}(CH_3)$	196, 252		
	15 - $C_{29}H_{59}(CH_3)$	225		
3130	11 - $C_{31}H_{63}(CH_3)$	169, 309	421	436
	13 - $C_{31}H_{63}(CH_3)$	192, 281		
	15 - $C_{31}H_{63}(CH_3)$	225, 253		

Table 4. Components of the paraffins recovered from whole body extracts of female FLS horn flies.

Kovats' Index	Structure	Observed Fragments m/e	M-15	M
2330	9 - $C_{23}H_{47}(CH_3)$	140, 141 224, 225	323	none
	11 - $C_{23}H_{47}(CH_3)$	168, 169 196, 197		
2530	11 - $C_{25}H_{51}(CH_3)$	168, 169 224, 225	351	none
	13 - $C_{25}H_{51}(CH_3)$	196, 197		
2550	11,15 - $C_{25}H_{50}(CH_3)_2$	168, 169 239	none	none
2730	11 - $C_{27}H_{55}(CH_3)$	168, 169 252, 253	379	none
	13 - $C_{27}H_{55}(CH_3)$	196, 197 224, 225		
	5 - $C_{27}H_{55}(CH_3)$	337	379	none
	7 - $C_{27}H_{55}(CH_3)$	309		
2930	11 - $C_{29}H_{59}(CH_3)$	168, 169 281, 282	407	422
	13 - $C_{29}H_{59}(CH_3)$	196, 197 252, 253		
	15 - $C_{29}H_{59}(CH_3)$	224, 225		
2950	11,15 - $C_{29}H_{58}(CH_3)_2$	168, 169 224, 225 238, 239 295	421	none

Table 4. Continued.

Kovats' Index	Structure	Observed Fragments m/e	M-15	M
	13,17 - $C_{29}H_{58}(CH_3)_2$	196, 197 266, 267		
3130	11 - $C_{31}H_{63}(CH_3)$	168, 169 308, 309	435	450
	13 - $C_{31}H_{63}(CH_3)$	196, 197 280, 281		
	15 - $C_{31}H_{63}(CH_3)$	224, 225 252, 253		
	11 - $C_{31}H_{63}(CH_3)$	168, 169 308, 309	435	none
3150	9,13 - $C_{31}H_{62}(CH_3)_2$	140, 141 210, 211 280, 281 352, 353	449	464
	11,15 - $C_{31}H_{62}(CH_3)_2$	238, 239 252, 253 322, 323		
	13,17 - $C_{31}H_{62}(CH_3)_2$	197, 198 266, 267 294, 295		
3330	11 - $C_{33}H_{65}(CH_3)$	168, 169 336, 337	463	478
	13 - $C_{33}H_{65}(CH_3)$	196, 197 308, 309		
	15 - $C_{33}H_{65}(CH_3)$	224, 225 280, 281		
3350	11,15 - $C_{33}H_{66}(CH_3)_2$	168, 169 238, 239 280, 281 350, 351	none	none

Table 4. Continued.

Kovats' Index	Structure	Observed Fragments m/e	M-15	M
	13,17 - $C_{33}H_{66}(CH_3)_2$	196, 197 252, 253 266, 267 322, 232		
3530	11 - $C_{35}H_{71}(CH_3)$	168, 169	491	none

respectively; but paraffins in flies less than 24 h old comprised approximately 85% of the total in both sexes. The total paraffins from FLS and KLS mixed sexes and FLS fecal material showed chromatographic proflies qualitatively similar to FLS males and females.

Florida wild strain

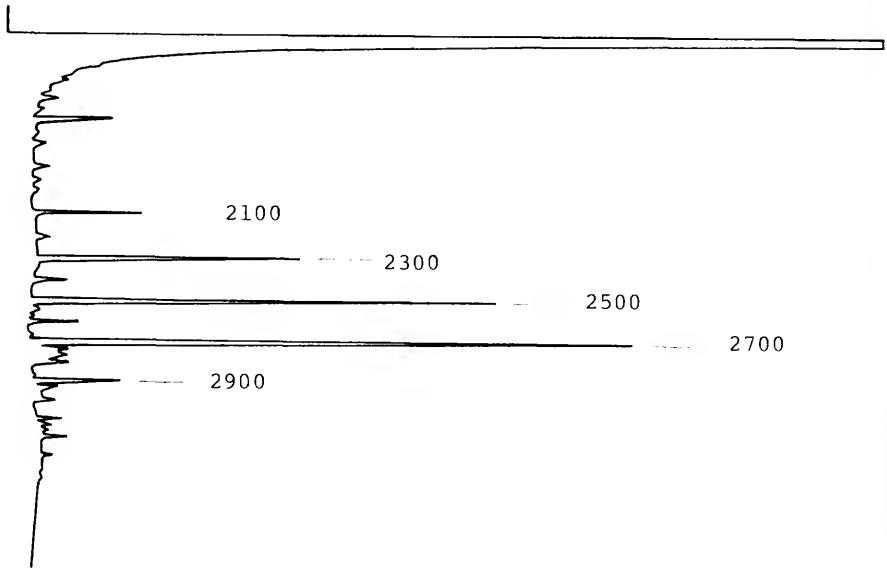
GC's of the male and female total paraffins are illustrated (Fig. 5). Quantitations of total paraffins and individual components are presented in Tables 1 and 2, respectively. FWS paraffins were not analysed by mass spectrometry; identification was based on comparing retention times of the eluting peaks with the identified FLS paraffins. This may be seen in comparisons between Fig. 4 and 5.

The paraffin components were generally as those observed with FLS flies, however there were significant quantitative differences between the 2 strains and between the FWS sexes. FWS males showed considerably more n-C_{21} and n-C_{23} than females; otherwise, the chromatographic profiles between the sexes were very similar.

House flies

GC's of the male and female house fly total paraffins are illustrated (Fig. 6). Unlike FLS horn flies, the male and female profiles differ considerably, particularly in regard to the methyl-branched components. Branched paraffins occurred in minute quantities in horn flies and male

Figure 5. Analytical GC's of total paraffins recovered from whole body extracts of virgin male (left) and female (right) 3-4 day old FWS horn flies.


2100
2300
2500
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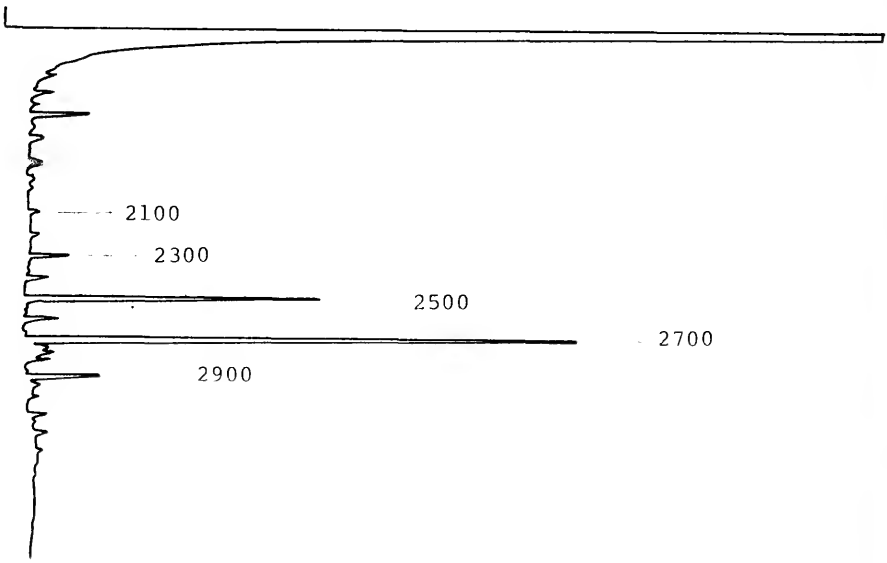
2100
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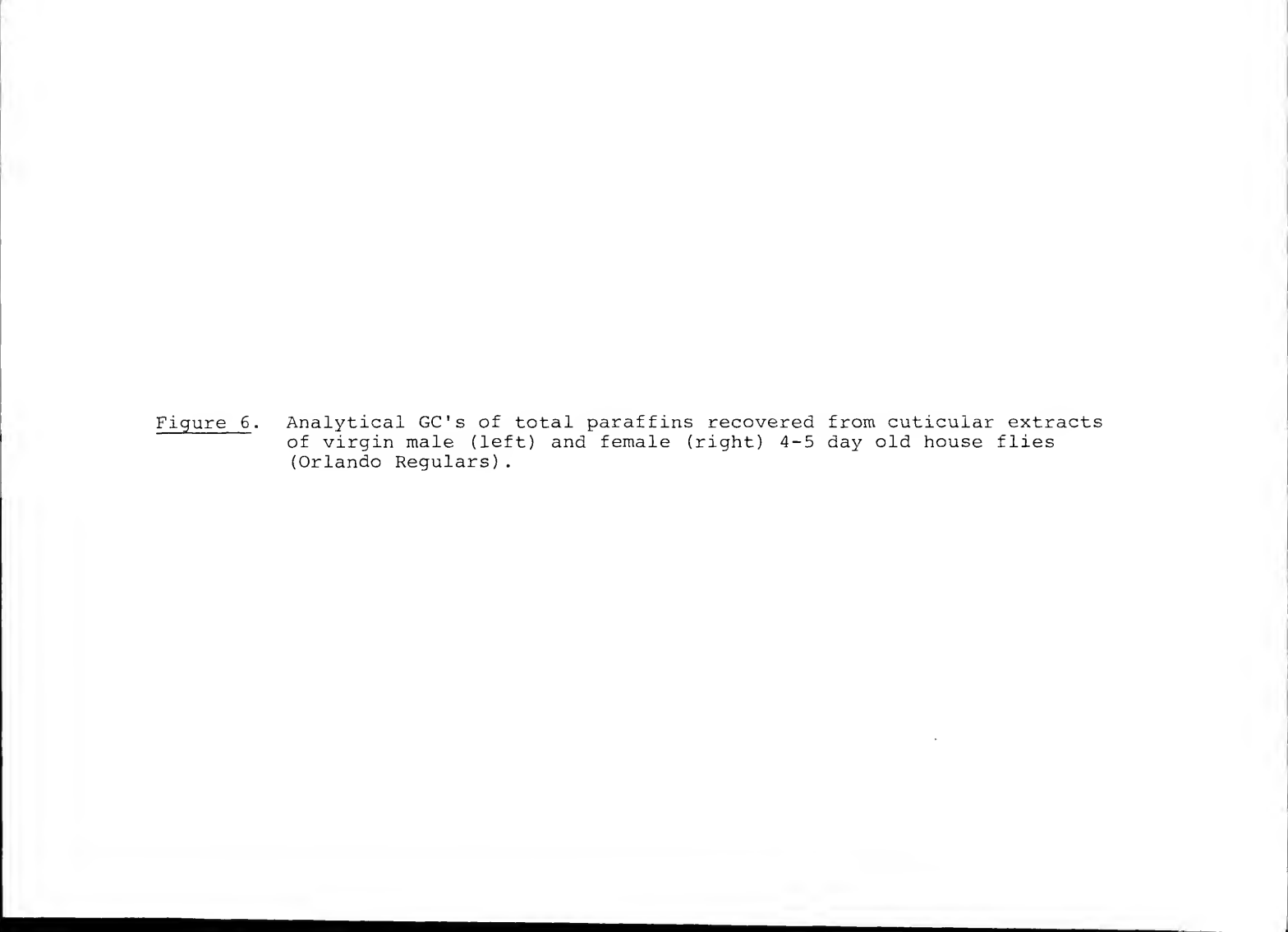
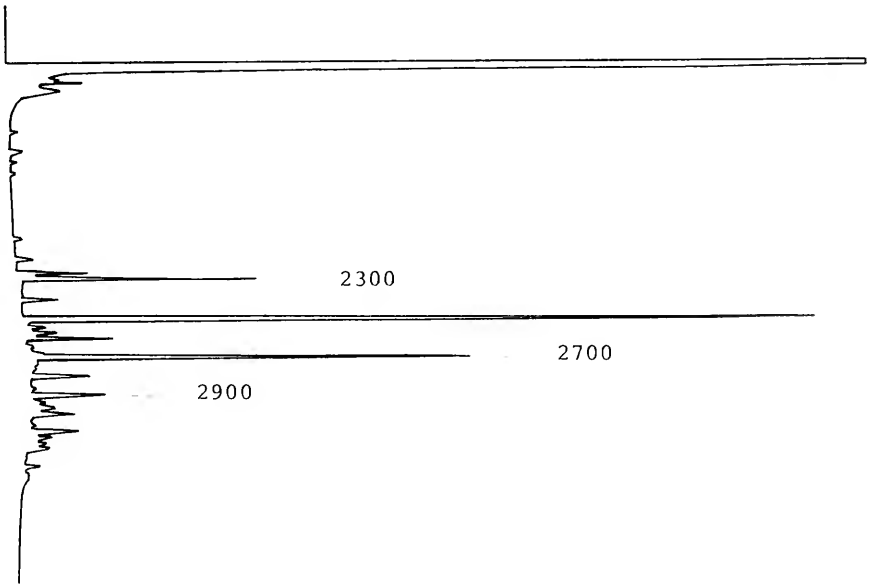
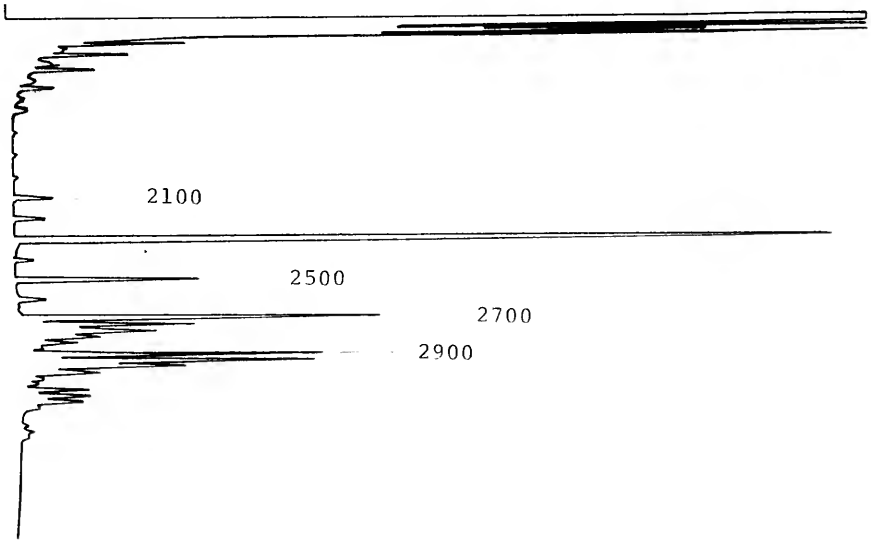


Figure 6. Analytical GC's of total paraffins recovered from cuticular extracts of virgin male (left) and female (right) 4-5 day old house flies (Orlando Regulars).


2300
2700
2900


2100
2500
2700
2900

house flies, but were much more abundant in female house flies. Uebel et al. (1976) found that by combining methyl- and dimethyl-branched C_{27} and C_{29} alkanes with muscalure, an enhancement of mating strike activity in house flies resulted. A complex of short-chain compounds (approximately C_8-C_{13}) occurred in both sexes (Fig. 6) but again much more abundantly in the females; the chemical nature and biological activity of these compounds has not yet been described.

Mono-olefins

Identification

Mono-olefins from all horn fly sources were found to be exclusively of the cis configuration. GC's of total mono-olefins recovered from FLS virgin flies, FWS virgin males, FWS virgin females, FLS and KLS mixed-sex flies, and FLS fecal material are illustrated in Fig. 5, 7, 8, 9, 10, and 11, respectively. Four major compounds occurred in each of the above sources, eluting at Kovats' indices 2272, 2292, 2477, and 2677, and designated I, II, III and IV, respectively. These compounds comprised 95% of the total mono-olefins in the FLS flies and FWS males, and 80% of the total in FWS females.

I and II were hydrogenated to a compound identical to n-tricosene (KI 2300) on an SE-30 column, thus implying lack of branching. Analysis of the hydrogenated II with EI/MS showed only a parent ion at m/e 324 and an otherwise

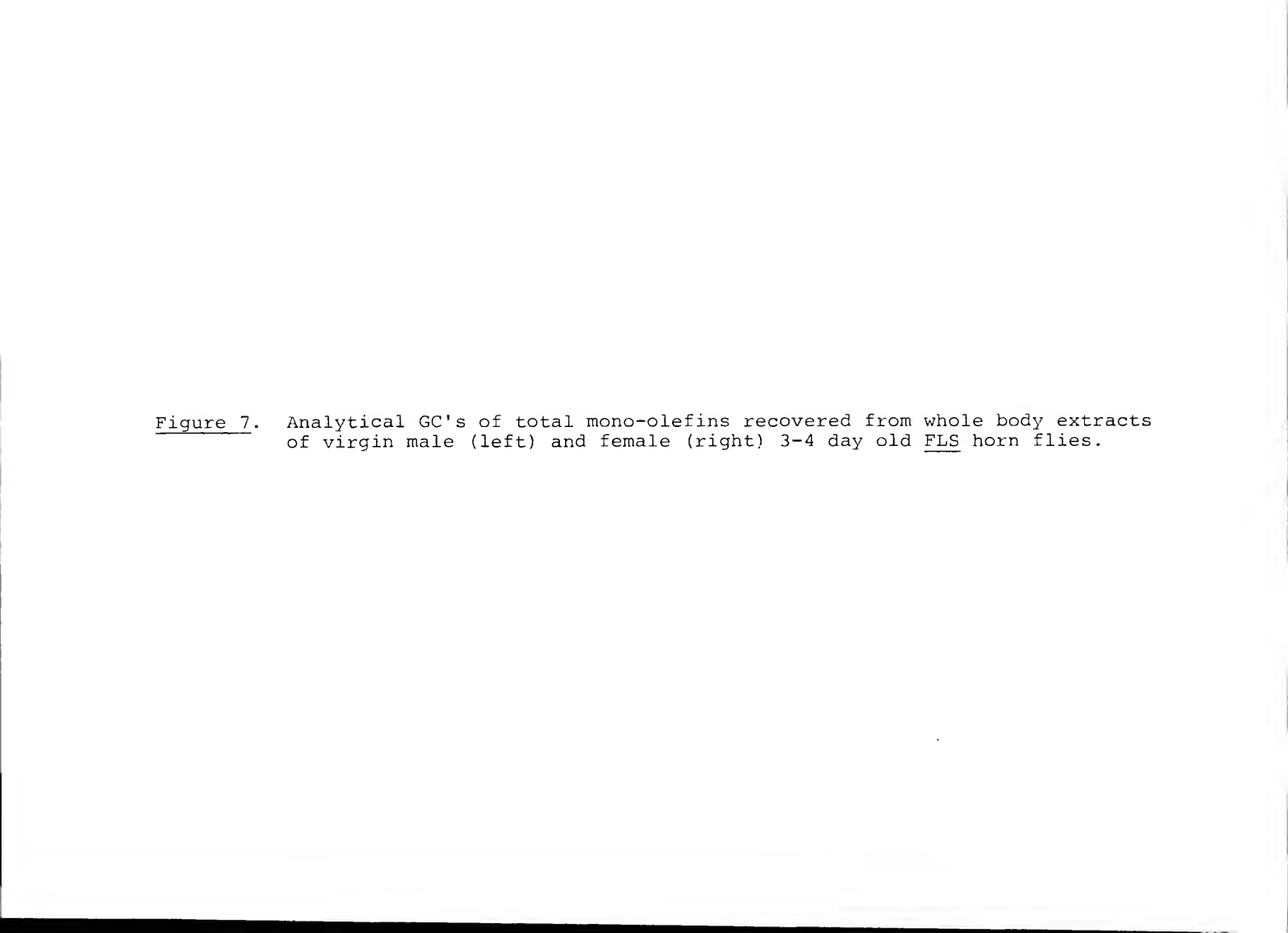
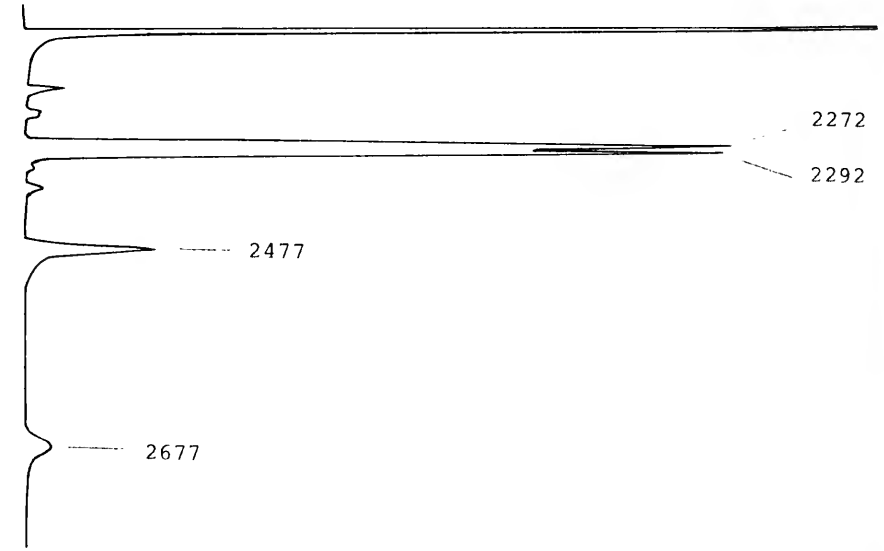
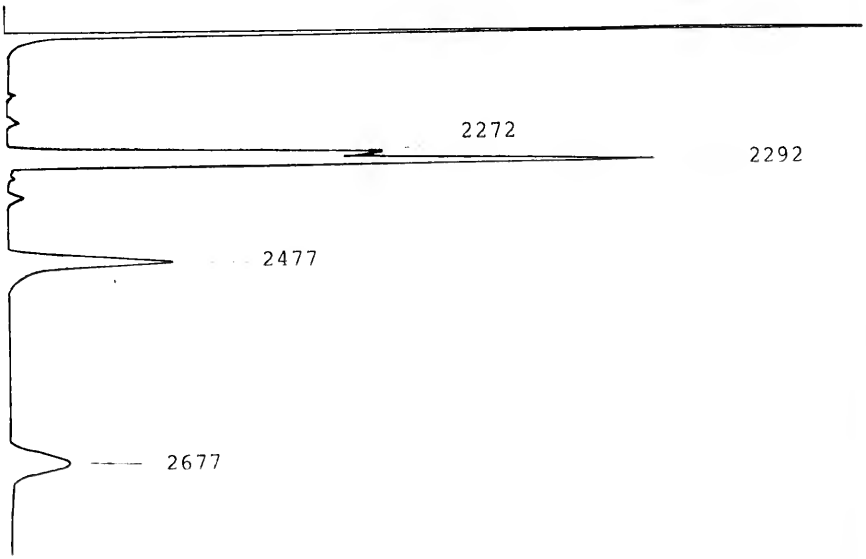


Figure 7. Analytical GC's of total mono-olefins recovered from whole body extracts of virgin male (left) and female (right) 3-4 day old FLS horn flies.


2272
2292
2477
2677


2272
2292
2477
2677

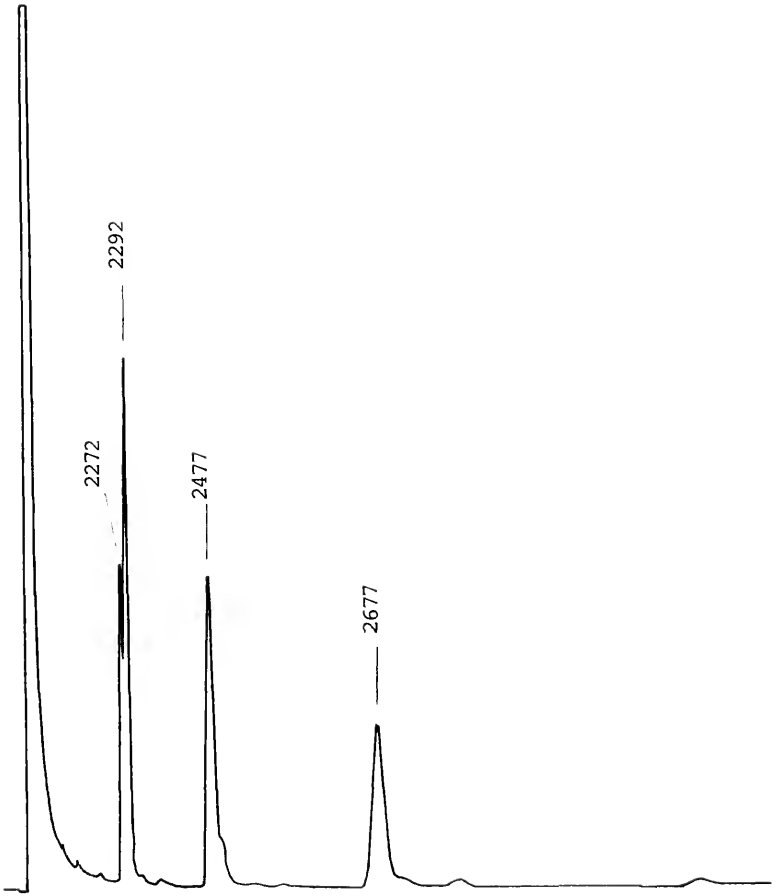


Figure 8. Analytical GC's of total mono-olefins recovered from whole body extracts of virgin males, 3-4 day old FWS horn flies.

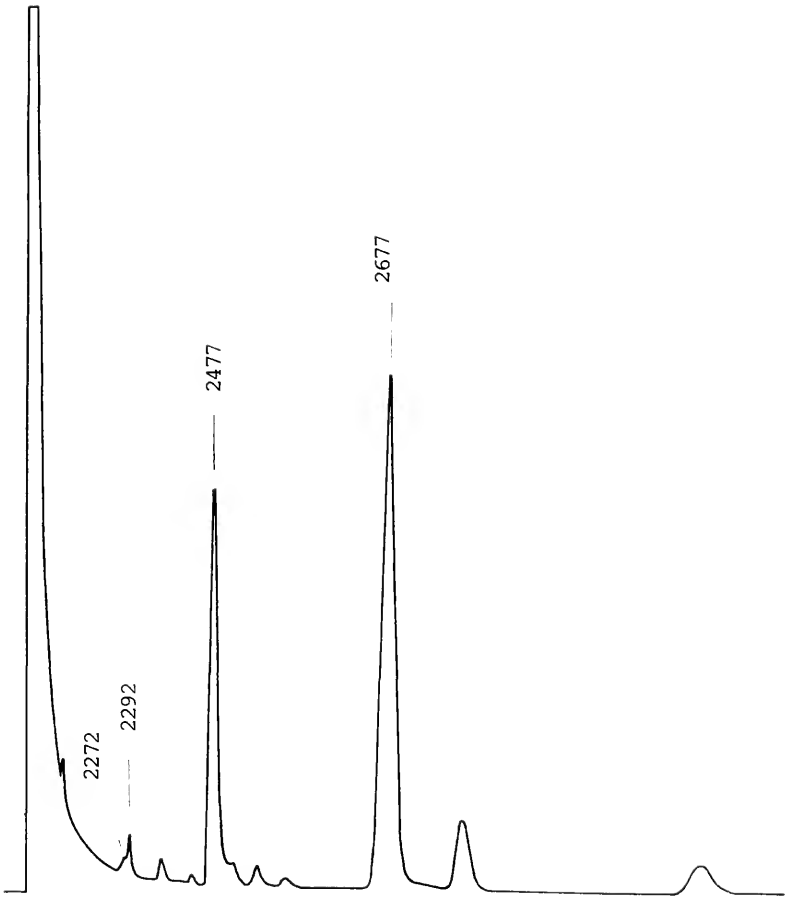


Figure 9. Analytical GC's of total mono-olefins recovered from whole body extracts of virgin female 3-4 day old FWS horn flies.

Figure 10. Analytical GC's of total mono-olefins recovered from whole body extracts of mixed-sex FLS (left) and KLS (right) 3-4 day old horn flies.

2272
2292
2477
2677

2272
2292
2477
2677

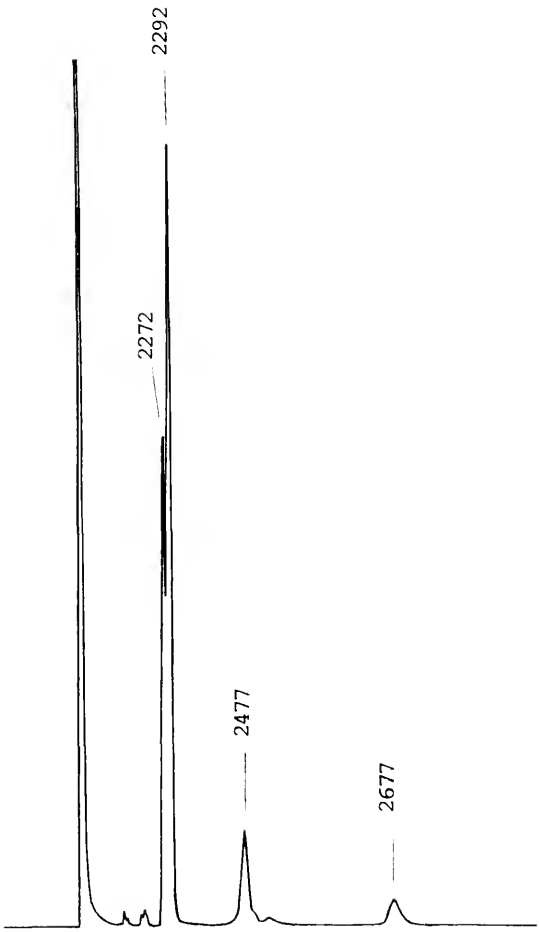


Figure 11. Analytical GC of total mono-olefins recovered from fecal extracts of FLS horn flies.

featureless spectrum giving no evidence for branching. Analysis of II (parent material) with CI/MS showed its molecular weight to be 322. Mass spectra, therefore, revealed the compound to indeed be a mono-olefin. Infrared spectra of II showed no vinyl absorption, implying location of the double bond toward the middle of the molecule.

The major longer aldehyde fragments resulting from ozonolysis of compounds I, II, III, and IV co-eluted on GC with C_{14}, C_{18}, C_{16}, and C_{18} aldehyde standards, respectively. The major shorter aldehyde fragments from ozonized III and IV co-eluted with the C_9 aldehyde standard. The shorter aldehyde fragments from ozonized I and II were not detected; this was due to either presence of contaminants or masking by the solvent front. Analysis by CI/MS confirmed the identity of the detected aldehyde fragments (Table 5). All of the major aldehydes had fragments M+1, M-1, M+1-18, and M-1-18; for some aldehydes, M+29 and M+41 fragment ions (addition of $C_2H_5^+$ and $C_3H_7^+$, respectively) were observed as noted. The presence of these aldehydes indicated the site of unsaturation to be at carbon number 9, 5, 9, and 9 for I, II, III, and IV, respectively. I, II, III, and IV co-eluted on GC with the synthetics Z-9-tricosene (muscalure), Z-5-tricosene, Z-9-pentacosene, and Z-9-heptacosene, respectively. The ozonolysis products of I and II co-eluted on GC with the ozonolysis products of their respective synthetics.

Table 5. Ozonolysis products of mono-olefins recovered from whole body extracts of female FLS horn flies.

Kovats' Index	Compound	Aldehydes	Observed Fragments M-1-18	M+1-18	M-1	M	M+1	M+29	M+41
2272	Z-9-tricosene (I)	C_{14}+	193	195	211	212	213	213	none
2292	Z-5-tricosene (II)	C_{16}	221	none	239	none	241*	269	281
		C_{17}	235	none	253	none	255	283	none
		C_{18}+	249	none	267	none	269	297	none
2477	Z-9-pentacosene (III)	C_{9}+	123	125	141	none	143*	171	183
		C_{13}	none	none	197	none	199*	227	none
		C_{14}+	none	none	211		213*	none	none
		C_{16}	221	223	239	240	241*	269	281
		C_{18}	249	none	269	none	269*	297	none
2677	Z-9- eptacosene (IV)	C_{8}	none	111*	none	none	129	157	169
		C_{9}+	123	125	141	none	143	171	183
		C_{15}	none	207	225	227	none	255	none
		C_{16}	221	223	239	none	241*	269	281
		C_{17}	235	237	253	254	255	283	295
		C_{18} +	249	251	267	268	269	297	309
		C_{20}	277	none	295	296	297	325	none

+Major products
*Base peak

In summary, the data show that at least 80% of the mono-olefins from each source (except newly emerged flies) consisted of C_{23}, C_{25}, and C_{27} straight chain molecules. Two positional isomers of tricosene were found with sites of unsaturation at the 9 and 5 positions. Penta- and heptacosene existed as Z-9 isomers. Z-9-tricosene has previously been identified as a house fly sex attractant pheromone (Carlson et al. 1971), and Z-9-pentacosene has been identified as the copulatory sex pheromone of the little house fly, Fannia canicularis (L.) (Uebel et al. 1975a).

Quantitations

Recoveries of hydrocarbons from FLS and FWS virgin horn flies are recorded in Table 1. Mono-olefins comprised 65% (1 FE = 5.13 µg) and 55% (1 FE = 7.17 µg) of the total hydrocarbons for 3-4 day old FLS males and females, respectively. Only 15% (1 male FE = 1.09 µg; 1 female FE = 0.59 µg) of the total hydrocarbons were mono-olefins in newly eclosed (less than 24 h old) FLS flies. Mono-olefins comprised only 35% (1 FE = 2.12 µg) and 30% (1 FE = 2.34 µg) of the total hydrocarbons for 3-4 day old FWS males and females, respectively.*

*The determinations of a given quantity of material weighed on the Mettler balance and computed on the integrator did not always agree. The higher quantities consistently recorded on the Mettler balance (eg, 1 FE FLS 3-4 day old females mono-olefins = 7.17 µg vs. 2.6 µg computed on the integrator) was due impart to contamination of samples by particles of silica gel and sodium sulfate from liquid chromatography.

Quantities of the major mono-olefins recovered from sexually mature FLS and FWS flies are summarized in Table 6. Tricosene (I and II), pentacosene (III), and heptacosene (IV) comprised 95% of the total mono-olefins for all strains of sexually mature flies, except FWS females, and the fecal extracts. In these cases, the relative quantities of I, II, III, and IV ranged from 12.8 to 46.5%, 25.1 to 48.2%, 8.8 to 29.5%, and 3.2 to 26.4% of the total mono-olefins, respectively. Tricosene was the major component, comprising 38-83% of the total; the 2 isomers of tricosene fluctuated in quantity and in proportion to one another depending on the source. Quantities of compounds I-IV from FWS females differed greatly from the other groups: Tricosene comprised less than 2% of the total mono-olefins while penta- and heptacosene comprised 22% and 55%, respectively; together these 4 compounds made up only 80% of the total mono-olefins and 2 other unidentified olefins of higher molecular weight made up the remainder.

The quantity of mono-olefins was roughly the same for sexually mature FLS males and females (1 FE = 2.41 μg for males and 2.56 μg for females - Table 2), although males possessed 17% more tricosene than females. Males possessed twice as much 9-tricosene but only 0.7 as much 5-tricosene as females. The proportion of 9-tricosene to 5-tricosene in males was 6:4, the ratio was exactly reversed in females.

FWS males and females also showed roughly similar quantities of mono-olefins (1 FE = 1.32 μg for males and 1.29 μg

Table 6. Quantitations of the major mono-olefins recovered from whole body extracts of 3-4 day old, male and female FLS (below) and FWS (opposite) horn flies.

Kovats' Index	Compound	µg of Component Recovered/Fly		% of Total Mono-olefins Recovered	
		Male	Female	Male	Female
2272	Z-9-tricosene (I)	1.20	0.64	46.5	23.8
2292	Z-5-tricosene (II)	0.76	1.04	29.5	38.5
2477	Z-9-pentacosene (III)	0.34	0.54	13.2	19.9
2677	Z-9-heptacosene (IV)	0.12	0.34	4.7	12.7
	Totals	2.42	2.56	93.9	94.9

Table 6. Extended.

Kovats' Index	Compound	µg of Component Recovered/Fly		% of Total Monoolefins Recovered	
		Male	Female	Male	Female
2272	Z-9-tricosene (I)	0.18	0.0089	12.8	0.6
2292	Z-5-tricosene (II)	0.35	0.0213	25.1	1.3
2477	Z-9-pentacosene (III)	0.42	0.37	29.5	22.6
2677	Z-9-heptacosene (IV)	0.37	0.896	26.4	55.1
	Totals	1.32	1.2962	93.8	79.6

for females - Table 2), but were only half that recorded for FLS males and females; FWS flies showed significantly greater quantities of penta- and heptacosene than FLS flies. FWS males, FLS females, and FLS males possessed 20, 55, and 64 times more tricosene than FWS females. Heptacosene was the majority component in FWS females, and it exceeded the quantities recorded from the other sources.

Newly eclosed FLS flies contained only 20% and 14% of the mono-olefin content of sexually mature males and females, respectively (Table 7). Only 34% and 27% of the mono-olefins for males and females, respectively, eluted with I, II, III, and IV. Tricosene made up of 11.4% and 3.7% of the total mono-olefins for males and females, respectively; these amounts were 2.8% and 0.7% of the tricosene found in sexually mature males and females, respectively.

Polyolefins

The 99:1 hexane-ether eluents from sexually mature, male and female FLS flies showed no evidence of polyolefins by TLC and GC (lower limit of detectability approximately 4 nanograms).

Olfactometer Assays

Response to Test Materials

Response of horn flies to test materials is recorded in Tables 8, 9, and 10. Summaries of these responses for female and male test insects are given in Tables 11 and 12, respectively.

Table 7. Quantitation of the major mono-olefins recovered from whole body extracts of newly-emerged (24 h old), male and female FLS horn flies.

Kovats' Index	Compound	µg of Component Recovered/Fly		% of Total Mono-olefins Recovered	
		Male	Female	Male	Female
2272 + 2292	Z-9-tricosene (I) + Z-5-tricosene (II)	0.054	0.012	11.4	3.7
2477	Z-9-pentacosene (III)	0.047	0.023	9.8	7.0
2677	Z-9-heptacosene (IV)	0.059	0.037	12.5	11.0
	Totals	0.16	0.072	33.7	21.7

Table 8. Response of horn flies[a] to test materials[b] in the modified 4-port olfactometer.

Test Materials	Amount	Sex of Test Insects	Reps	Total Insects Tested
Total Hydrocarbons (natural)	100µg	Male	8	316
		Female	10	389
Z-9-tricosene (synthetic)	50µg	Male	6	239
		Female	8	306

[a] 3-4 day old FLS virgins, 40 flies/rep.
[b] Natural extracts recovered from 3-4 day old FLS mixed-sexes.
[c] See text.

Table 8. Extended.

Response						Ratio	Statistics[c]	
Treatment			Check			(Trt:Ck)	Z-Test	Fisher's
No.	%	$\bar{x}$±SE	No.	%	$\bar{x}$±SE			
99	31.3	12.4±5.1	71	22.5	8.9±4.6	1.39:1	P<.001	P<.001
67	17 2	6.7±3.8	26	6.7	2.6±2.6	2.58:1	P<.001	P<.001
40	16.7	6.7±3.1	21	8.8	3.5±2.4	1.90:1	P=.01	P=.1
36	11.8	4.5±5.2	21	6.9	2.8±2.6	1.71:1	P=.025	P=.25

Table 9. Response of horn flies[a] to test materials[b] in the 4-unit olfactometer illuminated by the plant light.

Test Materials	Amount	Sex of Test Insects	Reps	Total Insects Tested
Total Mono-olefins (natural)	1mg	Male	25	996
		Female	25	992
Total Paraffins (natural)	1mg	Male	15	594
		Female	15	600
Z-9-tricosene (synthetic)	50µg	Male	4	151
		Female	18	717
	1mg	Male	16	627
Z-5-tricosene (synthetic)	1mg	Male	8	291
		Female	11	440

[a] 3-4 day old FLS virgins, 40 flies/rep.
[b] Natural extracts recovered from 3-4 day olf FLS mixed-sexes.
[c] See text.
[d] NS = Not significant at the P=.05 level.

Table 9. Extended.

Response						Ratio	Statistics[c]	
Treatment			Check			(Trt:Ck)		
No.	%	$\bar{x}$±SE	No.	%	$\bar{x}$±SE		Z-Test	Fisher's
64	6.4	2.6±2.0	63	6.3	2.5±4.1	1.02:1	NS[d]	-
170	17.1	6.8±4.9	101	10.2	4.0±2.9	1.68:1	P<.001	P=.01
48	8.1	3.3±2.1	60	10.1	4.0±2.5	0.8:1	NS	-
73	12.2	4.9±6.7	84	14.0	5.6±4.0	0.89:1	NS	-
17	11.3	4.3±4.0	18	11.9	4.5±5.1	0.94:1	NS	-
115	16.0	6.4±4.1	73	10.2	4.1±3.2	1.58:1	P<.001	P=.05
59	9.4	3.7±3.5	93	14.8	5.8±5.4	0.63:1	P=.005	P=.025
37	12.7	4.6±3.1	44	15.1	5.5±4.0	0.84:1	NS	-
33	7.5	3.0±3.1	36	8.2	3.3±3.6	0.92:1	NS	-

Table 10. Response of horn flies[a] to test materials[b] in the 4-unit olfactometer illuminated by red light.

Test Materials	Amount	Sex of Test Insect	Reps	Total Insects Tested	Response								Ratio (Trt:CK)	Statistics[c]	
					Treatment				Check					Z-test	Fisher's Test
					Number	%	X	± SE	Number	%	x	± SE			
Male Crude Lipid (natural)	20 FE	Male	16	320	101	31.7	6.3	± 4.5	90	28.1	5.6	± 3.6	1.12:1	NS	-
		Female	16	320	144	45.0	9.0	± 4.7	77	24.1	4.8	± 2.9	1.87:1	P<.001	P<.001
Male Crude Lipid (Natural)	20 FE	Male	16	320	127	39.7	7.9	± 4.4	118	36.9	6.9	± 5.2	1.08:1	NS	-
		Female	16	320	149	46.6	9.3	± 4.9	88	27.5	5.5	± 4.4	1.69:1	P<.001	P<.001
Mixed-Sex Non-hydrocarbon. Crude Lipid (Natural)	20 FE	Male	16	320	140	43.8	8.8	± 4.6	120	37.5	7.5	± 4.0	1.17:1	NS	-
		Female	16	320	104	32.5	6.5	± 4.8	109	34.1	6.8	± 3.7	0.95:1	NS	-
Mixed-Sex Monoolefins (natural)	1 mg	Male	18	360	180	50.0	10.0	± 5.2	132	36.6	7.3	± 4.7	1:36:1	P=.005	P<.001
		Female	10	200	103	51.5	10.3	± 2.8	64	32.0	6.4	± 2.7	1.64:1	P=.005	P<.01
Z-9-tricosene (synthetic)	1 mg	Male	16	320	118	36.9	7.5	± 4.4	169	52.8	10.6	± 4.2	0.70:1	P=.005	P<.001
		Female	15	300	123	39.7	8.2	± 5.1	128	41.3	8.5	± 5.0	0.96:1	NS	-
Z-5-tricosene (synthetic)	1 mg	Male	32	640	291	45.5	9.1	± 4.4	186	29.1	5.8	± 3.8	1.56:1	P<.001	P<.001
		Female	16	320	131	40.9	8.2	± 4.8	134	41.9	8.4	± 4.1	0.98:1	NS	-
Z-9:Z-5 tricosene 60:40[d] (synthetic)	1 mg	Male	16	320	149	46.6	9.2	± 4.1	125	39.1	7.8	± 3.9	1.20:1	NS	-
		Female	16	320	120	37.5	7.5	± 3.9	124	38.8	7.8	± 4.1	0.97:1	-	-
Z-9:Z-5 tricosene 40:60[e] (synthetic)	1 mg	Male	16	320	145	45.3	9.1	± 3.8	123	38.4	7.7	± 3.3	1.18:1	NS	-
		Female	16	320	142	44.4	8.9	± 3.8	133	41.6	8.3	± 4.2	1.07:1	NS	-
Z-9-pentacosene (synthetic)	1 mg	Male	16	320	116	36.3	7.3	± 3.5	128	40.0	8.0	± 4.2	0.91:1	NS	-
		Female	16	320	126	39.1	7.9	± 4.5	90	28.1	5.6	± 3.7	1.4:1	P=.01	P<.001
Z-9-heptacosene (synthetic)	1 mg	Male	16	320	127	39.7	7.9	± 4.2	145	45.3	9.1	± 4.4	0.88:1	NS	-
		Female	16	320	109	34.1	6.8	± 5.2	136	42.5	8.5	± 5.2	0.8:1	P=.05	-

a/ 3-4 day old FLS virgins, 20 flies/rep
b/ Natural extracts recovered from 3-4 day old FLS mixed-sexes or virgins
c/ See text
d/ Reflects ratio of these 2 isomers in male flies
e/ Reflects ratio of these 2 isomers in female flies
f/ NS = not significant at the P.05 level

Table 11. Summary of responses of female horn flies to test materials in the olfactometers.

Test Material	Amount	Conditions[a]	Total Response[b] (%)	Ratio (Trt:Ck)	Z-Test	Fisher's
Female Crude Lipid	20FE	RL	69.1	1.87:1	P<.001	P<.001
Male Crude Lipid	20FE	RL	74.1	1.67:1	P<.001	P<.001
Nonhydrocarbons	20FE	RL	66.6	0.95:1	NS[c]	-
Total Hydrocarbons	100µg	4-Port	23.9	2.58:1	P<.001	P<.001
Total Mono-olefins	1mg	RL	83.5	1.61:1	P=.005	P=.01
	1mg	PL	27.3	1.68:1	P<.001	P=.01
Total Paraffins	1mg	PL	26.2	0.89:1	NS	-
Z-9-tricosene	50µg	PL	26.2	1.58:1	P<.001	P=.05
	50µg	4-Port	18.6	1.71:1	P=.025	P=.25
	1mg	RL	83.7	0.96:1	NS	-
Z-5-tricosene	1mg	PL	15.7	0.92:1	NS	-
	1mg	RL	82.8	0.98:1	NS	-
Z-9:Z-5 (60:40)	1mg	RL	76.3	0.97:1	NS	-
Z-9:Z-5 (40:60)	1mg	RL	85.9	1.07:1	NS	-
Z-9-pentacosene	1mg	RL	67.5	1.4:1	P=.01	P<.001
Z-9-heptacosene	1mg	RL	76.6	1.25:1	P=.05	-

[a] RL: "Red Light", 4-unit olfactometer
PL: Plant Light, 4-unit olfactometer
4-Port: Modified 4-port olfactometer
[b] Treatment + Check/Total Flies Tested
[c] NS: Not significant at P=.05

Table 12. Summary of responses of male horn flies to test materials in the olfactometers.

Test Material	Amount	Conditions[a]	Total Response[b] (%)	Ratio (Trt:Ck)	Z-Test	Fisher's
Female Crude Lipid	20FE	RL	58.7	1.12:1	NS[c]	-
Male Crude Lipid	20FE	RL	76.6	1.08:1	NS	-
Nonhydrocarbons	20FE	RL	81.3	1.17:1	NS	-
Total Hydrocarbons	100µg	4-Port	53.8	1.39:1	P<.001	P<.001
Total Mono-olefins	1mg	PL	12.6	1.02:1	NS	-
	1mg	RL	86.7	1.36:1	P=.005	P<.001
Total Paraffins	1mg	PL	18.2	0.8:1	NS	-
Z-9-tricosene	50ug	PL	23.2	0.94:1	NS	-
	1mg	PL	21.1	0.63:1	P=.005	P=.025
	50µg	4-Port	25.5	1.9:1	P=.01	P=.1
	1mg	RL	89.7	0.7:1	P=.005	P<.001
Z-5-tricosene	1mg	PL	27.8	0.84:1	NS	-
	1mg	RL	74.5	1.56:1	P<.001	P<.001
Z-9:Z-5 (60:40)	1mg	RL	85.6	1.2:1	NS	-
Z-9:Z-5 (40:60)	1mg	RL	83.8	1.18:1	NS	-
Z-9-pentacosene	1mg	RL	76.3	0.91:1	NS	-
Z-9-heptaccsene	1mg	RL	85.0	0.88:1	NS	-

[a] RL: "Red Light", 4-unit olfactometer
PL: Plant Light, 4-unit olfactometer
4-Port: Modified 4-port olfactometer
[b] Treatment + Check/Total Flies Tested
[c] NS: Not significant at P=.05

Statistical analysis

Two statistical methods were applied to the data: (1) the Z-test, and (2) Fisher's method of combining probabilities from independent replicates (Anderson and Bancroft 1952). In a given test, when the difference between treatment and check was significant at $P<.05$ using the Z-test, the data were also analyzed using Fisher's method. Since the 2 methods differed in approach it was felt a broader analysis of the data was achieved.

The Z-test determined differences in the proportion of responding insects entering treatment and check choice chambers in any given test (eg, Table 10 - Female Crude Lipid (natural) and Female Test Insects: Treatment = 144 and Check = 77). The test statistic was calculated as follows:

$$Z = P_t - P_o \;/\; \sqrt{P_o\ q\ /n}$$

where, P_t = Treatment/Treatment + Check

$P_o = 0.5$

$q = 1 - 0.5$

n = Treatment + Check

and the level of significance then determined.

Using Fisher's method, the bionomial probability (P_i) for each replicate in a given test was calculated. A given probability was based on a comparison between the number of insects responding to treatment and the number responding to treatment + check (i.e., the total number of responding

insects). The bionomial probabilities of a given test were combined according to the following expression:

$$-2 \Sigma \log_e P_i$$

at df = 2 x number of replicates, and the level of significance then determined.

Response of females

Female flies responded positively (i.e., treatment significantly greater than check) to both male and female crude lipids, total hydrocarbons, and total mono-olefins (Table 11). The only mono-olefin to demonstrate a significant response over check was Z-9-pentacosene (Tables 10 and 12 - $P = .01$; $P' < .001$). Responses to nonhydrocarbons and total paraffins were not significantly greater than response to checks ($P = .05$); responses to Z-5-tricosene and the various combinations of tricosene isomers (60;40 and 40;60 mixtures of Z-9 and Z-5 observed in male and female horn flies, respectively) were not significant. Z-9-tricosene generated confusing responses: responses were positive but low probability levels were obtained with the modified 4-port olfactometer ($P = .025$; $P' = .25$); highly significant positive results were obtained with the 4-unit olfactometer PL ($P < .001$; $P' = .05$); and nonsignificant results were obtained with the 4-unit olfactometer with RL. The only reversal (check treatment) recorded was a significant response ($P = .05$) to the check against Z-9-heptacosene, implying some repellency.

Although significant responses were elicited in many cases, positive responses to the horn fly crude and fractionated lipids were generally considered weak based on treatment:check ratios. Of the eight positive responses recorded, the average treatment:check ratio was only 1.77:1.

Response of males

Bioassays failed to clearly define the response of male flies to the various horn fly lipids (Table 11). Responses to male and female crude lipids, the nonhydrocarbons, and the total paraffins were not significantly greater than checks ($P = .05$).

Response to the total mono-olefins and tricosene was not conclusive. Positive responses were obtained when the total mono-olefins were tested under RL but not PL conditions. Responses to check exceeded treatment when 1 mg Z-9-tricosene was tested under RL andPL conditions; response to treatment was positive (but not significant - $P' = .1$) when tested with the modified 4-port olfactometer; response to 50 ug Z-9-tricosene under PL was not significant compared to checks. Z-5-tricosene elicited a positive response under RL conditions but not under PL conditions. Various combinations of the tricosene isomers (Z-9 and Z-5) failed to elicit positive responses, as did Z-9-penta- and heptacosene. Once again, where positive responses occurred they were generally weak in comparison with responses seen in other insect species.Treatmentcheck ratios ranged from 0.63 to

1.9:1, with reversals occurring in 2 instances with Z-9-tricosene (0.7:1 and 0.63:1).

Extraneous Factors Affecting Response

Light

Light greatly influenced the response of horn flies in the olfactometers. "Red light" (RL) generated greater upward movement than the plant light (PL) in the 4-unit olfactometer (Tables 9, 10, 11, and 12: % Response). Flies were readily attracted to the white light of the PL impinging on the holding chambers and upward movement out of the chambers was greatly reduced. The neutral response to red light permitted negative geotactic and positive olfactory responses to take over and greater upward movement resulted.

The degree of positive phototaxis exhibited by horn flies was illustrated by Morgan (1966) when he employed a UV lamp to attract flies off a host; this indicated a greater response by horn flies to visible rather than olfactory stimulation. Similar findings have been reported by workers studying chemical communication in positively phototactic insects: Adams and Mulla (1968), working with the eye gnat, Hippelates collusor, found they could greatly reduce the number of nonresponders in an olfactometer by eliminating light trapping effects. Shorey and Gaston (1965) showed that Trichoplusia ni males normally responded to pheromone in the absence of competing light stimuli; however, in the

presence of sufficient light, a phototactic response became dominant and males were attracted to light in lieu of the pheromone.

Fly response in trials employing "double blanks" revealed choice chamber preferences due to position effect. In the 4-unit olfactometer employing RL, fly response in tests ranged from 30% to 50% among the choice chambers. The range of response with PL was 2.5% to 12.5%. For unknown reasons, position effect among the choice chambers shifted from time to time. In the modified 4-port olfactometer which consisted of one set of 2 choice chambers, response was a third greater in the right chamber. The preferred chamber was situated slightly closer to the light source than its counterpart and this may be the reason for the position effect. In both olfactometers, differential light impingement on choice chambers was concluded to be responsible for chamber preferences.

Sex of test insect

Response of males and females differed with respect to the type of olfactometer and type of light employed. In the modified 4-port olfactometer, female response to double blanks was only 40% that of males (16%/chamber for males vs. 6.7%/chamber for females). In the 4-unit olfactometer with PL, female response exceeded that of males by 60% (11.2%/chamber for females vs. 7.1%/chamber for males); but with RL, response was equal (44%/chamber).

Photoperiod

Initially, tests were conducted when convenient for the author, but it was noted that response tended to decrease in tests run late in the day (4-7 PM). Subsequently, testing was arbitrarily scheduled between the hours of 10 AM and 3 PM in an attempt to reduce any possible effects of photoperiod.

Flight behavior of the horn fly, which may affect response in an olfactometer, is known to be influenced by photoperiod. Tugwell et al. (1966) found greater flight activity in early morning and with a greater percentage of females. Hargett and Goulding (1962) showed that when lights in the colony were turned off, large numbers of flies left the host. In the case of house fly sexual activity, Parker (1962) and Murvosh et al. (1964) indicated that a diurnal rhythm may be operative; this observation prompted Mayer and Thaggard (1966), who were investigating house fly pheromones, to state the need for controlling photoperiodic effects when assaying pheromones. Chaudhury et al. (1972) found significant changes in responsiveness of the face fly when conducting bioassay tests of fly extracts between 9 AM and 6 PM. But Lodha et al. (1970) reported that the face fly exhibits a distinct diurnal mating rhythm which is unimodal. Later on, Chaudhury and Ball (1974) concluded that a daily rhythm of sex pheromone release and/or production is most probably present in virgin female face flies.

Restricting the time of testing was believed to help neutralize any photoperiodic effects. Under RL conditions and a darkened room (4-unit olfactometer), no effects were observed other than the increased response of flies; it was believed the change in response under these conditions was related to horn fly visual acuity and not alterations of photoperiod.

Design of olfactometer

Even slight differences in design affected response as witnessed in the results obtained from the modified 4-port and 4-unit olfactometers (Tables 8 and 9: Response).

In constructing the 4-unit olfactometer, attempts were made to satisfy the requirements of the horn fly and the stable fly so that both could be tested with the same apparatus. Naturally, compromises in the design were necessary. The horn fly exhibits a strong negative geotaxis (Hargett and Goulding 1962) which perhaps deserves greater attention when considering design; Kinzer et al. (1970) minimized the effects of gravity by employing a horizontal design. In my study, such a design may have improved treatment: check response ratios.

Response ratios may have been improved by lengthening the holding chambers and therefore enlarging the reaction area (the area below choice ports in the holding chamber). An enlarged reaction area may have permitted test insects greater room to maneuver when orientating towards choice ports.

Cross-contamination of check and treatment air streams can disrupt response. Daykin and Kellogg (1965) and Kinzer et al. (1970) overcame this problem in their olfactometers by completely separating check and treatment air streams. In the 4-unit olfactometer, cross-contamination was not observed; a smoke trail revealing the air stream (Fig. 3) showed adequate separation of the air streams until they reached the lower portion of the holding chamber. Horizontal olfactometers may have problems of stratification of chemicals within the test chamber due to differential weight of the molecules.

Other factors

Observations made during olfactometer trials indicated that degree of starvation of test insects, outside weather conditions, pheromones in test insects, and host affinity may have influenced response of test insects. Data are incomplete since no tests were conducted to specifically evaluate these factors; action taken to reduce possible bias contributed by some of these factors was therefore based on limited subjective observations.

On a given day of testing, blood meals for test insects were initially made available only during the morning before testing commenced. Flies became more starved as the day progressed, and a decrease in response was noted with flies tested later in the day. To standardize the blood-fed condition, blood meals thereafter were made available to flies up to 1 h before they were tested.

Weather characterized by low barometer (eg, afternoon thundershowers) appeared to bring about a reduction in fly activity. Similar reductions in activity were noted in olfactory tests with house flies (M. S. Mayer, pers. comm.). Again, to eliminate this possible source of bias, tests were discontinued at the onset of bad weather.

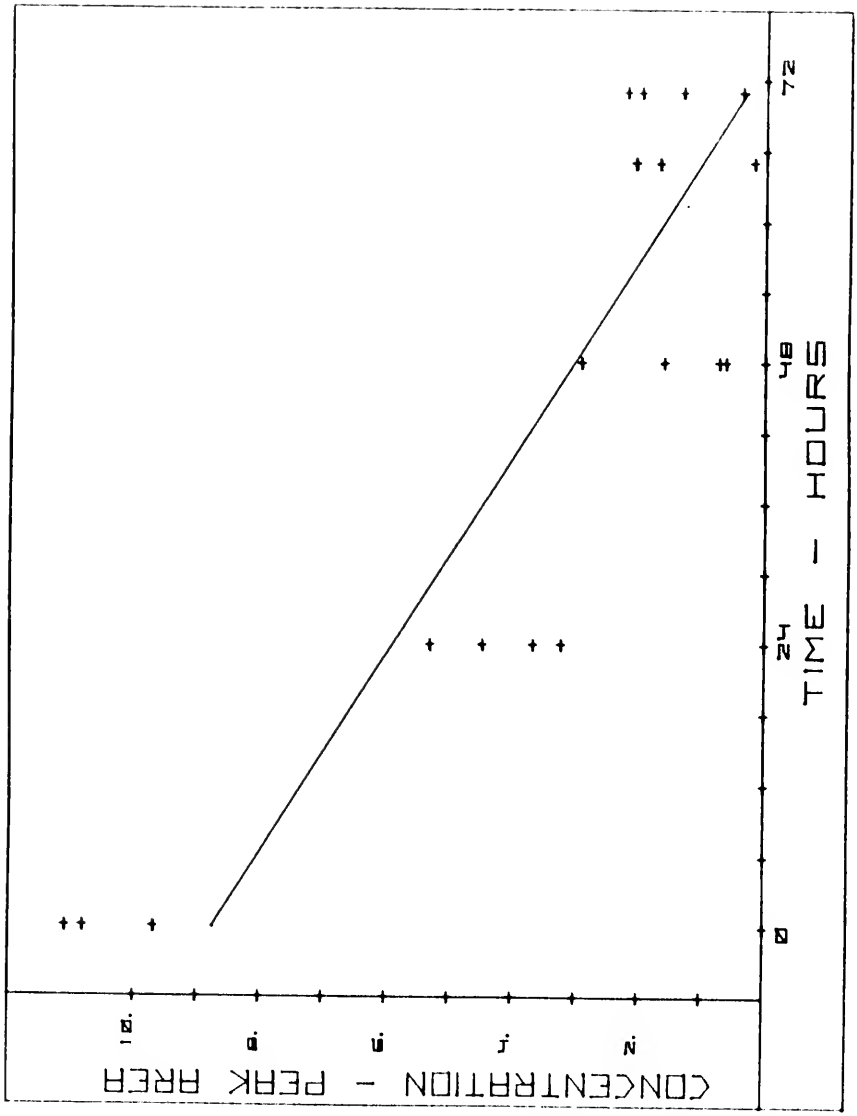
Active components in the test insects themselves may have affected response to test materials. For instance, where female test insects are attracted to female crude lipids, the test insects could possibly become disoriented because they are affected by 2 sources of material. If any disorientation occurred, a decrease in response would be expected.

In nature, the adult horn fly remains closely associated with its host; even when away from the host, the fly is engaged in migratory or questing flights directed at searching out a new host. This host finding drive may take priority and mask responses to a pheromone in an olfactometer. However, the host finding drive may be altogether absent in laboratory strain flies, having been selected out by the long series of bottlenecks experienced during maintenance of the FLS colony.

Evaporation Rates of Muscalure

The regression line and equation of muscalure loss (100 µg deposit) on exposure time is presented (Fig. 12). After 72 h exposure in the 4-unit olfactometer, over 95% of the

Figure 12. Regression of muscalure concentration on exposure time, 100 μg initial deposit. $y = 8.7287 + (-0.1187)x$, $R^2 = 0.797$.


CONCENTRATION - PEAK AREA
TIME - HOURS
0
24
48
72

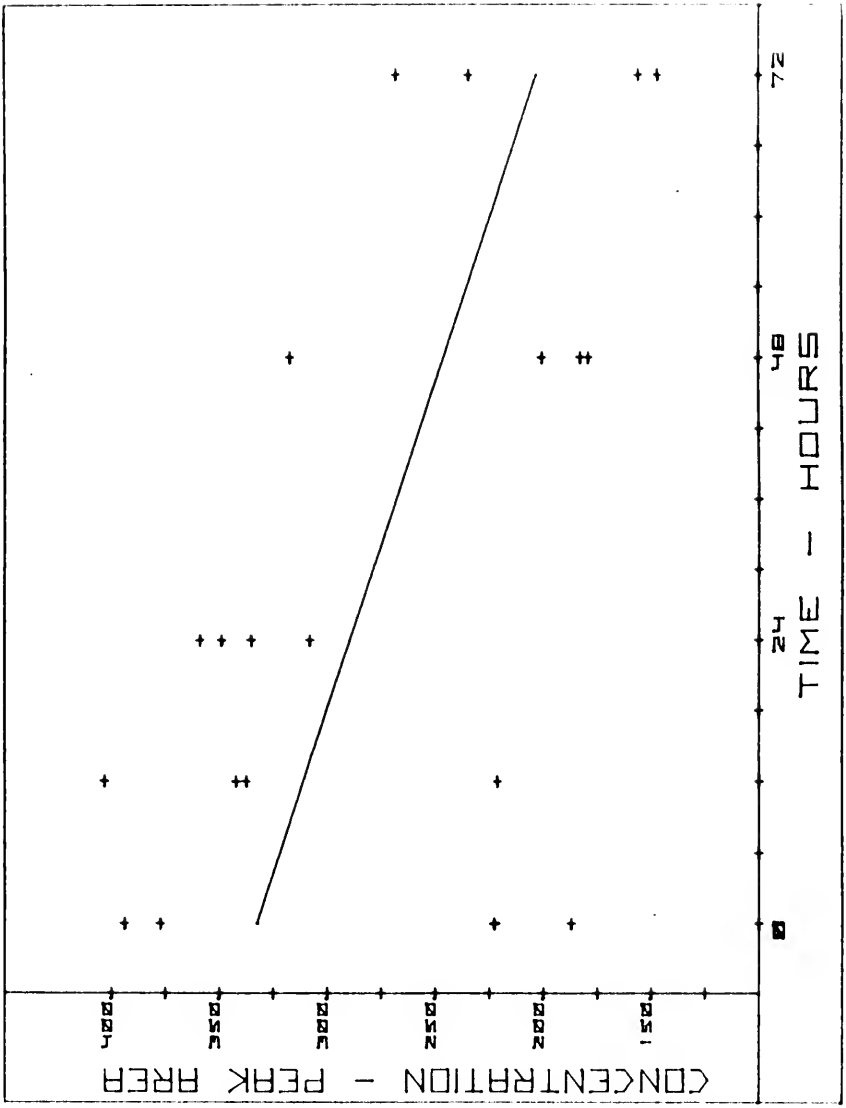
deposited muscalure had evaporated, or at the rate of 6.8 µg/30 min. Extreme variations in the loss rates from the 1 mg deposit (R^2 = 0.314, Fig. 13) precluded any conclusions.

Other studies conducted to determine evaporation rates of muscalure show similar results. In laboratory olfactometers, Carlson (pers. comm.) found 1.58 µg muscalure/30 min to evaporate from filter papers treated with 100 µg; using the same olfactometers, greater response from house flies was achieved when muscalure was applied to glass plates, thus implying rates of evaporation greater than from filter paper (Carlson 1971). Under highly variable field conditions, Carlson and Beroza (1973) showed the muscalure loss rate averaged 3.62 µg/30 min from treated corn cob grits.

Contact Stimulation Assays

The mating activity is characterized by observations briefly described as follows: The responding test fly usually approached the impaled fly from underneath; initially the legs would meet and entangle with pronounced grasping and stroking. If contact did not break at this point, the responding fly would eventually begin crawling all over and intensely probe the impaled fly; sometimes the responding fly would return to its initial position underneath the impaled fly and repeat the leg contact activity. At times as many as 5 test flies would engage in these activities at once, and in some cases contacts persisted for 30 seconds or more. Simultaneous responses by many flies and prolonged contacts were particularly evident with male flies exposed

Figure 13. Regression of muscalure concentration on exposure time, 1 mg initial deposit. $y = 332.0877 + (-1.7939)x$, $R^2 = 0.314$.


CONCENTRATION - PEAK AREA
TIME - HOURS
0
24
48
72

to live or dead female flies. Live impaled flies rarely beat their wings, but displayed much leg and head movement activity; responding flies often beat their wings when maneuvering under the impaled fly. Attempts at mounting and copulation were few, and only occurred with male test flies exposed to dead impaled female flies. There was little or no monosexual behavior displayed among test flies.

Tables 13 and 14 summarize the bioassays for contact stimulation. A live impaled female fly evoked the greatest response from male test flies (22.5±14.97 contacts/30 min., 15 test males; 40.4±32.7 contacts/30 min., 25 test males); a dead female evoked roughly half the response of a live female (8.6±9.39 contacts/30 min., 15 test males; 19.2±18.24 contacts/30 min., 25 test males); and a dead female thoroughly washed in hexane drew no response at all. A live impaled male fly elicited no response from male test insects. Male flies failed to respond significantly to dead male flies and pseudoflies treated with various female extracts, despite the evidence of good removal of active material(s) from female flies with hexane.

A live impaled male elicited a response on the part of female test flies (4.5±0.7 contacts/30 min., 15 test females; 6.6±5.56 contacts/30 min., 25 test females). However, female response to male flies was only 15-20% that of male response to female flies.

Table 13. Response[a] of male horn flies[b] to variously treated female and male horn flies[b] and pseudoflies[c].

Treatment	Reps	Number of Test Males	Contacts[d] $\bar{x}$ SE
Live Female	24	15	22.5 ± 14.97
	25	25	40.4 ± 23.7
Dead Female	9	15	8.6 ± 9.39
	23	25	19.2 ± 18.24
Female Washed with Hexane	10	25	0
Live Male	6	15	1.0 ± 1.09
Pseudofly + 10 FE Female Crude Lipid	2	15	0
Pseudofly + 100-300 µg Female Mono-olefins	6	25	0.13± 0.35
Dead Male + 2 FE Female Crude Lipid	6	25	0
Dead Male + 50 µg Z-9-tricosene	10	25	0.2 ± 0.4
Dead Male + 50 µg Z-5-tricosene	4	25	1.0 ± 1.41

[a] Contacts lasting 3 seconds or longer; 30 min. tests in 20 ml beaker.
[b] 3-4 days old, virgin.
[c] Fly-size knot cut from black thread.
[d] All treatments differed significantly (P = .05, t-test) from the "Live Female" treatment for 15 and 25 test males, respectively.

Table 14. Response[a] of female horn flies[b] to female and male horn flies[b].

Treatment	Reps	Number of Test Females	Contacts[c] $\bar{x}$ · SE
Live Male	2	15	4.5 ± 0.71
	19	25	6.6 ± 5.56
Live Female	2	15	1 ± 0
	12	25	1.75 ± 1.77

[a] Contacts lasting 3 seconds or longer; 30 min. tests in 20 ml beaker.
[b] 3-4 days old, virgin.
[c] Response to "Live Male" and "Live Female" was significantly different (P = .05, t-test) for 15 and 25 test females, respectively.

DISCUSSION

General Response of Test Insects

High variability characterized the response of test insects in the olfactometer and contact stimulation assays (note $\bar{x} \pm$ S.E., Tables 8, 9, 10, 13, and 14). The possible sources of this variation have been discussed (Results: Extraneous Factors Affecting Response). Under similar test conditions, response of this nature is typical of other muscoid species such as the house fly (Cowan and Rogoff 1968; Mayer 1971; Mayer et al. 1972) and the stable fly (J. W. Mackley, unpublished data). Extensive replication and a multiple statistical analysis were necessary to insure valid results from highly variable response data.

In the olfactometers, 1 mg of test material was generally employed when assaying a given fraction or compound because it elicited the most uniform response; test materials shown to be positive at 1 mg concentration rarely showed positive responses at smaller concentrations (eg, 50-100 µg). The marginal nature of response and the failure to demonstrate dose-response relationships reflect an unrefined bioassay and/or the lack of a strong response by horn flies to their lipid components. Thus, the olfactometer assay provided only

gross indications of activity, i.e., either a positive or neutral response, and was unable to delineate degrees of response. An olfactometer assay of horn fly response has been attempted only once before (Kinzer et al. 1971); here, responses to human and cow emanations were similar to responses observed in this study, i.e., averaged 2:1.

More work is necessary to develop an olfactometer system which will improve treatment-check ratios and measure response differences in relation to dose. Systems with these capabilities are necessary for studying muscoid attractants.

Paraffins

Methyl-Branched Components

Paraffins recovered from other muscoid species have not been shown to be attractive in olfactometer assays (i.e., not attractive over distance). However, in the cases of the house fly (Uebel et al. 1976) and the tsetse fly, Glossina morsitans (Carlson, pers. comm.), certain methyl-branched paraffins are active as mating stimulants as demonstrated in pseudofly tests. In this study, the assessment of mating or contact stimulation by test materials was precluded due to the failure to establish a complete contact assay, i.e., test males responded to females but not to pseudoflies and other fly models in contact assays. As a final alternative, the chemical nature of the horn fly methyl-branched paraffins was assessed to see what comparisons could be made with the active components found in house and tsetse flies.

The quantities of C_{27} and C_{29} methyl-branched components found in female house flies far exceeded those found in laboratory and wild strain horn flies. The role of these methyl-branched paraffins in enhancing the mating stimulant effect the muscalure in house flies has been demonstrated (Uebel et al. 1976). Di- and trimethyl-branched paraffins such as those in the stable and tsetse fly were not found in the horn fly, while paraffins beyond the range of C_{31} occurred in small quantities or were altogether absent in the horn fly. Based on the small quantities of methyl-branched paraffins found in the laboratory and wild strain horn flies, it is questionable that they play a role similar to what has been described with house flies, stable flies, and tsetse flies. In addition, muscalure is found in both sexes of FLS horn flies, so its role in conjunction with branched paraffins to trigger mating or contact stimulation is unclear. Combinations of paraffins with muscalure were not tested in the olfactometers.

Unbranched Components

Quantitatively and qualitatively, FLS male and female straight-chain paraffins showed no significant differences. Absence of differences between sexes suggests an absence of sexually-based contact stimulation; but lack of a suitable assay prevented biological testing.

Significant quantitative differences between some corresponding straight-chain paraffins in FWS males and females indicates a possible source of biological activity. Uebel et

al. (1975c) have shown that although male and female face flies secrete the same active olefins, the higher quantities of nonacosane and heptacosane in mature males was responsible for attenuating active male alkenes. In another fly species, Fannia femoralis (Stein), addition of female paraffins increased the activity of olefins (Uebel et al. 1977b), while tests with Z-9-tricosene added to n-paraffin mixtures and natural paraffins had no effect or decreased response of male house flies (Carlson, unpublished data). Given the differences between male and female n-paraffins, a similar mode of action may be operating in wild horn flies. However, owing to the lack of material and a suitable assay, FWS flies and extracts were not tested.

Mono-olefins

Response

Olfactometer studies revealed positive but low level activity centered at the mono-olefins. Only females registered consistent responses, but female response to individual synthetic components was confusing because different results were obtained from different olfactometers and lighting conditions. Male response was irregular; however, data indicate that male activity may be similar to female activity but at a lower level. Results may indicate that attractancy involves a mixture of components as seen in fruit flies (Nation 1975). Many of the current investigators are concluding that

this is more widely the case in pheromone-producing insects (Wright 1964; Law and Regnier 1971; Mayer and McLaughlin 1975).

Quantitation

The significant quantitative differences between FLS and FWS flies and between FWS males and females may indicate areas of biological activity. For example, the near lack of tricosene in wild females and its presence in wild males may suggest attraction of females to males;in olfactometer tests, laboratory strains of house flies show the opposite case where males are attracted to female-produced tricosene (Carlson et al. 1971). The profile of female FWS mono-olefins may reflect the laboratory diet to which the flies were exposed, but the same would be expected of wild males which in fact more closely resembled FLS flies. In any event, the differences found in mono-olefin content between FLS and FWS flies prevent direct application to the field of biological or chemical data acquired from FLS flies.

Increases in mono-olefins and concurrent decreases in paraffins occurred with age in FLS flies (Tables 1, 2, and 3). Investigating house fly cuticular hydrocarbons, Silhacek et al. (1972a) reported that although the chief function of hydrocarbons is restricting water loss, it was unlikely that compositional changes in female hydrocarbons were a response to this function. Instead, these workers suggested that changes in cuticular hydrocarbons are more closely associated

with female sexual activity, and that these chemical changes possibly function to alter the rate of attractant release. Changes in hydrocarbon content occurred in both sexes of FLS horn flies, but the increase in mono-olefin content may be a function of sexual activity as suggested.

Fecal Material

Similarities between fecal and body mono-olefins suggests a common origin. Silhacek et al. (1972b) stated that hydrocarbons in the cuticular lining of the gut may be deposited in the feces or that hydrocarbons evaporated from the cuticle later condense on fecal contaminated surfaces; attractants released this way could be distributed over wider areas and not be restricted to site of production. A horn fly attractant could be released and distributed in this manner on the surface of the host animal.

Strain Differences

The most striking strain differences were noted between laboratory and wild flies. Only differences in chemical nature were determined; lack of material prevented any behavioral studies.

Given the history of the colonized strain (FLS, KLS) the differences in relation to the FWS were not unexpected. Several severe bottlenecks have been experienced in establishing and maintaining the laboratory colony. The FLS is a product of 11 viable eggs recovered from 75,000 F_1,

laboratory-reared FWS flies (Greer 1975). The colony has experienced periodic slumps, usually during the winter months, almost every year for the past 8 years; during these slumps the number of individuals has been reduced to several hundred.

The effects of inbreeding and the consequent diminution of the gene pool are obvious. Establishment of the colony has shown that behavior characteristics relating to host affinity, mating rituals, feeding and diet, and likely others, had to be first selected out or greatly altered (Wilkerson 1974; Butler, unpub. data). Conditions under which the colony has been maintained may have had little selective value on sex attraction and/or aggregating behavior, whereas natural selection for such factors may be more pronounced in a natural population. These problems are universal in insect colonization. Similar differences have been noted between wild laboratory strains of house flies with respect to production of and response to muscalure (Carlson and Beroza 1973; Morgan and Gilbert 1973; Carlson, unpub. data).

Cross Attraction

The presence in the horn fly of substances identified as attractants and/or mating stimulants in other muscoid species is not an unusual phenomenon. Among a number of lepidopteran species, interspecific attraction has been demonstrated based on utilization of a common pheromone (Berger and Canerday 1968; Roelofs and Comeau 1970; Roelofs

and Tette 1970; Roelofs and Cardé 1971); and in muscoid flies, Fannia femoralis (Stein) and F. pusio (Wiedemann) not only produce the same mating stimulant pheromone, but males and females of opposite species have been observed attempting to copulate (Uebel et al. 1977b). Schneider (1962) pointed out that strict species specific activity of pheromones is not required, and that differences in other aspects of courtship behavior prevent interspecific mating. The cross attraction observed by Mayer et al. (1972) of male house flies to horn fly cuticular hydrocarbons was no doubt due to the presence of Z-9-tricosene.

Host-Parasite Relationships

Studies on the biology of the horn fly show that in nature host animals are primarily responsible for aggregating flies from over short distances, and that vision is the principle means of host detection. Once on the host, a set of factors serve to further restrict the fly to only certain areas of the host (Review of Literature: Bionomics). Factors directing long range and/or migratory flights are unknown, but it is unlikely that horn fly mono-olefins are involved as attractants over considerable distances owing to their low volatility.

The tsetse fly is another blood-feeding muscoid which heavily relies upon an animal host. After failing to demonstrate an airborne sex attractant, a number of investigators concluded that these tsetse flies regard the host as not

only a food source, but as a focus for meetings between the sexes (Bursell 1961; Dean et al. 1969).

Thus, it appears that the specific host-parasite relationships which exist for these fly species eliminate the need for a special sex attractant which works over distance. In a review of dipterous pheromones, Shorey (1973) found that their effect in causing attraction over a distance "ranged from very weak down to undetectable." He concluded that congregation of the sexes was mainly effected by response to non-pheromone stimuli, especially environmental aggregation stimuli.

Evaporation Rates of Muscalure

Data show that muscalure has a very low volatility and suggests it is biologically active in minute quantities. This assessment probably can be extended to the biologically active horn fly olefins.

The substrates upon which the muscalure is deposited may influence evaporation rates. Too few controlled tests were conducted to make any definite conclusions, but the limited tests indicate more rapid rates of evaporation from glass slides than from filter paper.

In the laboratory, highly variable evaporation rates from heavier deposits (1 mg) on glass slides may indicate a breakdown in the direct relationship between amount of deposit and amount of evaporation. This direct relationship is always assumed when determining dose-response curves to

confirm a sensory response (after Stevens 1971). In light of these variable evaporation rates, perhaps in the future data on evaporation rates should accompany dose-response data.

Contact Stimulation Assays

Positive responses by males toward live and dead females and a total lack of response towards hexane-washed females suggests that horn fly mating behavior is in part chemically mediated. Total lack of response by males towards other males and variously treated pseudoflies and a reduced response towards dead unwashed females indicate that vision also plays a role in mating behavior. Chemical tests may have been inconclusive due to other essential factors such as characteristic movements and sound production which may partially account for response differences observed between live and dead females. That a combination of visual, chemical, and possibly tactile stimuli are involved in sex recognition and/or mating stimulation in other muscoids has been demonstrated (Murvosh et al. 1964; Lodha et al. 1970; Tobin and Stoffolano 1973b; Langley et al. 1975; Muhammed 1975).

Visual perception appears to be more acute in the horn fly than in other muscoids based on the lack of courtship advances by males towards other males and pseudoflies. Male monosexual behavior is not uncommon in house flies (Murvosh et al. 1964; Tobin and Stoffolano 1973b), stable

flies (Muhammed 1975; Mackley, unpub. data), and other Diptera (Shorey 1973).

The response of females towards males show females to exhibit a very low degree of sexual aggressiveness. Dead males washed with hexane or unwashed were not tested, therefore this possibility exists that chemicals were responsible for mediating this behavior.

SUMMARY AND CONCLUSIONS

Olfactometer assays of horn fly lipid components showed laboratory strain female horn flies to be weakly attracted to natural horn fly mono-olefins. Four compounds made up 94% of the mono-olefins recovered from male and female laboratory reared flies: Z-9-tricosene (muscalure), Z-5-tricosene, Z-9-pentacosene, and Z-9-heptacosene. Tricosene was the major component in laboratory flies and wild male flies; heptacosene was the majority component in wild female flies. Z-9-tricosene and Z-9-pentacosene have been identified as sex pheromones in two other muscoid species. Assays of synthetics of the 4 components failed to show any single center of activity; however, only one concentration was generally tested. The weak response elicited by the flies to the attractant, coupled with the evidence from other investigators that the host animal is the chief means of aggregation, suggest that horn flies do not heavily rely on pheromones to be attracted over distance.

Paraffins were not found to be active in olfactometer assays but were chemically analyzed. The bulk of components were straight-chain molecules C_{21} to C_{29}; minor components consisted of mono- and di-methyl branched molecules and straight-chain molecules outside the C_{21} to C_{29} range. Active paraffins present in the house fly, stable fly, and

tsetse fly occurred in minute quantities in the horn fly or were altogether absent.

The olfactory assays were unrefined. The laboratory olfactometers were only able to register gross indications of activity and were unable to delineate degrees of response. Lack of refinement was in part due to a number of extraneous factors which affected response, the most notable being light.

Wild strain horn flies showed GC profiles quite different from laboratory strain flies, the most striking difference being the almost complete lack of tricosene in wild strain females. These chemical data suggest that there may be behavioral differences between laboratory and wild strains.

Contact stimulation assays showed that horn fly mating activity is in part chemically mediated. Lack of response by males towards variously treated fly models indicated that vision also plays a role in mating behavior. Assays for activity of fly extracts and synthetic compounds was not possible because of this lack of response by males to fly models.

The study of evaporation rates of muscalure in the olfactometer indicated that only a small portion of an applied test material will enter an air stream to affect test insects. Although only 2 concentrations were assayed, results indicated that evaporation rates are more variable at higher concentrations and that a direct relationship between amount of deposit and amount of evaporation of a test material does not necessarily exist.

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BIOGRAPHICAL SKETCH

James Weldon Mackley was born February 12, 1946, at Gettysburg, Pennsylvania. He graduated from Sykesville High School, Sykesville, Maryland, in June 1963. In September 1963, he entered the University of Maryland, College Park, Maryland, and graduated in June 1967, receiving a Bachelor of Science degree in Entomology.

In September 1967, he entered the Graduate School of the University of Florida. He was awarded a Master of Science degree in Entomology in June 1969.

He entered the U.S. Peace Corps in August 1969 and served 3 years in Thailand as a Malaria Entomologist assigned to the Thai Public Health Ministry. In April 1973 he returned to the United States and in September of that year he resumed graduate study for his doctorate in Entomology at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Reece I. Sailer
Graduate Research Professor
of Entomology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephen G. Zam
Associate Professor of
Microbiology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1977

Dean, College of Agriculture

Dean, Graduate School

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jerry F. Butler, Chairman
Associate Professor of
Entomology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harvey L. Cromroy
Professor of Entomology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard S. Patterson
Adjunct Associate Professor
of Entomology